# DISTRIBUTION OF IONOMOTIVE PUMPS AND CONTROL OF MALPIGHIAN TUBULE SECRETION BY CYCLIC NUCLEOTIDES IN THE BLACK-LEGGED TICK, *IXODES SCAPULARIS*

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**Abstract**

Ticks have a hematophagous feeding strategy, and thus must regulate the uptake of excess ions and fluids during blood meal engorgement through ion and osmoregulation. The salivary glands are essential for tick hydromineral balance as they play a vital role in excretion of excess ions and fluids back to the host. In order to further characterize organs involved in ion and osmoregulation, we examined activity of $Na^{+}/K^{+}$ ATPase and V-type $H^{+}$ ATPase, as these ionomotive pumps drive transport within the tick salivary glands and Malpighian tubules of other hematophagous insects. $Na^{+}/K^{+}$ ATPase and V-type $H^{+}$ ATPase were immunolocalized to the midgut, salivary glands, Malpighian tubules, intestine and rectal sac. We examined secretion rates of Malpighian tubules when stimulated by cAMP and cGMP, which stimulated tubule secretion. Future studies should examine putative diuretic factors in the regulation of these organs in order to further characterize their contribution to hydromineral balance.

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# CHAPTER 1:

# BIOLOGY OF IXODID TICKS AND THE ROLE OF TRANSPORTERS IN IONO- AND OSMOREGULATON IN BLOOD FEEDING DISEASE VECTOR ARTHROPODS

## **Introduction**

### Tick Taxonomy and Lifecycles

Ticks are obligate blood-feeding arthropod vectors of infectious diseases as they are able to transmit a variety of protozoa, rickettsiae, spirochetes, helminth parasites and bacteria (Bera 2009). Next to mosquitos, ticks are one of the main arthropod vectors of infectious diseases affecting humans, domestic animals, and livestock worldwide (Dantas-Torres, Chomel, and Otranto 2012). Ticks are arachnids of the suborder Ixodida, existing in three families – Argasidae, Nuttaliellidae, and Ixodidae (Oliver 2017). There are currently 867 known tick species, one species existing in the Nuttaliellidae family, while 183 exist as Argasids, and the remaining 683 exist as Ixodid ticks (Bera 2009). Species of the family Argasid are known as soft ticks due to their soft exterior, in comparison to Ixodid ticks which have a hard exocuticle. Ixodid ticks are mainly known for their medical and veterinary importance due to their role as vectors of a variety of diseases; however, previous reports have found that soft ticks can also cause infestation and medical ailments in both humans and animals during a blood meal (Bera 2009). Specifically, a variety of species belonging to the generas of *Argas*, *Ornithodoros* and *Otobius* possess the ability to cause disease in animals and humans. For example, Argasid ticks can be vectors of arboviruses such as West Nile in birds, African swine fever virus in domestic swine, and the spirochetes that cause relapsing fever in humans (Randolph 2000). Many species under the genera *Ornithodoros* are involved in the epidemiology of tick borne diseases, such as relapsing fever caused by spirochete *Borrelia* which causes fever, chills, aches, and nausea, and can continue for months if untreated (Estrada-Peña and Jongejan 1999). Argasids live in environments with hot temperatures, with a life cycle consisting of egg, larval, nymphal, and adult stages (Oliver 2017). Depending on the species, there are usually 2-4 nymphal instars,

which feed rapidly on several hosts before ecdysing to the next stage (Oliver 2017). Argasids are usually found in nests and burrows of birds, rodents, and in rural areas occupied by humans, and bites by species of this family can cause irritation, blisters, and bruising (Estrada-Peña and Jongejan 1999). The third family, which makes up 80% of tick species, is known as Ixodidae, and like other blood feeding arthropods, they are capable of transmitting a large variety of infectious agents (Hughes 2005). Ixodid ticks exist in four life stages, as eggs, larvae, nymphs and adults. Unlike Argasids, the Ixodid nymphal stage only consists of one instar (Oliver 2017). In addition, species of the Ixodid family must engorge for several days as they require larger blood meals in comparison to Argasids (Oliver 2017). Ixodid ticks go through a 3-host life cycle in which they engorge on mammals such as birds, deer, dogs, and humans after which they ecdyse from larva, to nymph, to adult (Hoogstraal and Aeschlimann, 1982). Like Argasid ticks, species of the Ixodid family thrive in spring and summer temperatures, engorging on small to large mammals and reptiles (Oliver 2017). Adult female Ixodidae usually mate on the host, then feeds to engorgement, detaches and lays eggs (Bera 2009). The remainder of this chapter will specifically focus on Ixodid ticks.

Ixodid ticks were first recognized as potential vectors of infectious diseases at the end of the 19$^{th}$ century by Smith and Kilbourne, who were able to show that a tick species, *Boophilus annulatus*, is able to transmit *Babesia bigemina*, a protozoan agent that causes Texas cattle fever (Sonenshine and Roe 2014). In addition to transmitting a variety of pathogens to human and animals, there is an increase in incidences of tick-borne diseases. Most diseases initially present nonspecific symptoms such fever or fatigue, and thus are difficult to diagnose. Additionally, there are a lack of diagnostic and clinical tests available (Ringdahl 2001). Through their hematophagous feeding strategy, ticks can cause a variety of medical ailments, such as paralysis,

toxicosis, allergies, and irritations (Bera 2009). Ticks are responsible for more than 100, 000 cases of human illness, and are the most important disease vectors in domestic and wild animals (Fuente et al. 2008). Among other blood feeding vectors, such as *Aedes aegypti* and *Rhodnius prolixus,* ticks transmit the largest variety of microparasites to humans and animals; including fungi, viruses, bacteria, and protozoa (Randolph 2000). Various species of Ixodid ticks are known to be vectors of *Rickettsia rickettsii,* a bacterium which causes Rocky Mountain spotted fever, a bacterial disease resulting in fever and headaches in humans (Dantas-Torres 2007). The pathogen can be maintained through transovarial and transstadial passage, with the former meaning it can be passed from an infected adult female to her eggs, and the latter meaning it can be maintained throughout developmental life stages (Dantas-Torres 2007). *Dermacentor variabilis,* the American dog tick, is known to be the primary vector of this bacterium in the United States, while in Canada, *Dermacentor andersoni* is the primary vector (Dantas-Torres 2007). Rocky Mountain spotted fever is one of the most virulent infections to humans, causing cutaneous, cardiac, pulmonary, gastrointestinal, renal, and neurological symptoms which can be fatal (Dantas-Torres 2007). Mediterranean spotted fever, a tick-borne disease caused by *Rickettsia conorrii,* carried by the brown dog tick, was first discovered in 1910. Mediterranean spotted fever is found in southern and central Europe, North Africa, India, and central and southern Africa (Rovery, Brouqui, and Raoult 2008). Both Rocky Mountain spotted fever and Mediterranean spotted fever, like various vector diseases, have a high mortality rate, and often go undiagnosed and untreated due to diagnostic and clinical testing failures. Lyme disease is also often undiagnosed or misdiagnosed often due to false positive and false negative serology tests. The antibody for *B. burgdorferi* appears between two to six weeks after the onset of Lyme. If a serology test is done before this, results may be suggestive of no Lyme, even though the reality is

otherwise. In addition, serology tests may produce false positives due to the presence of another spirochetal infection like syphilis or rheumatoid diseases (Gill et al. 2005). Initially, Rocky Mountain spotted fever presents the classical clinical triad of fever, headache, and rash and therefore is difficult to diagnose. In addition, the antibody for Rocky Mountain spotted fever does not appear until 10 days after disease onset (Dantas-Torres 2007). Clinical presentations of tick-borne diseases are often ambiguous common symptoms resulting in their under and mis-diagnosis.

The list of possible diseases and infections caused by varying species of ticks differs globally depending on pathogen and tick distribution. Tick-born rickettsioses and protozoan parasites carried by the vector cause serious diseases and infections. In addition, bites by ticks can also cause both allergic and toxic reactions (Estrada-Peña and Jongejan 1999). Over the last two decades, the number of tick-borne diseases affecting humans has increased along with the rate of mortality and morbidity caused by these emerging diseases (Troughton and Levin 2007).Tick infestations also impact livestock, not only by increasing mortality through disease, but also causing an economic impact by reducing animal weight and milk production (Fuente, Kocan, and Blouin 2007). The ability for ticks to transmit diseases to livestock, places further restraint on livestock survival and production, causing economic loss, particularly in both tropical and subtropical areas of the world (Bera 2009). Tick-borne protozoan diseases such as theileriosis and babesiosis, along with rickettsial diseases such as anaplasmosis, cowdriosis, and tick-associated dermatophilosis, contribute to both medical and economical problems of livestock (Zahid Iqbal et al. 2006). In tropical and subtropical areas, livestock are directly affected by ticks through their feeding strategy as there is a huge loss of blood. Such blood loss causes a decrease in weight and can also lead to anemia, or dermatitis, if blood sucking occurs by

large numbers (Zahid Iqbal et al. 2006). Economic loss from livestock also occurs indirectly through tick infestations due to their roles as vectors for pathogens and toxins (Zahid Iqbal et al. 2006). A tick bite can lead to paralysis and also cause physical damage to the livestock through irritation and unrest. Due to their importance as vectors of infectious diseases, and their incidences in both medical and veterinary clinical settings, there is a need to develop methods for supressing and controlling ticks to reduce pathogen transmission.

***Ixodes scapularis* as vectors of Lyme disease**

Lyme disease is the most common tick-borne systemic infectious disease to be reported in the United States (Falco et al. 1999; States et al. 2015). In Canada, Lyme disease incidences increased six fold over a period of six years between 2009 to 2015 (Gasmi et al. 2017). Lyme is caused by the spirochete *Borrelia burgdorferi* and is transmitted to both humans and animals through various ticks of the genus *Ixodes.* Of reported cases in the United States, 90% are from northeast, mid-atlantic, and upper-midwest United States, where the vector for *B. burgdorferi* is *I. scapularis,* also known as the black-legged tick (Falco et al. 1999). *Ixodes scapularis* are hard ticks of the Ixodid family, and aside from serving as prominent vectors of Lyme disease, it is also a vector for other human diseases including human granulocytic anaplasmosis, and babesiosis, which both present as symptoms of fever, chills, nausea, and muscle aches lasting months (Ribeiro et al. 2006). In areas where the spirochete, *B. burdorferi,* and its vector, *I. scapularis,* are common, there can be as many as 50-100 incidences of the disease per 100,000 inhabitants in the area (Franz and Krause 2003). Lyme is also very common in areas of Europe, where the primary vector for the spirochete there is *Ixodes ricinus* (Franz and Krause 2003).

Lyme disease most often begins with a bullseye skin rash, referred to as erythema migrans. Symptoms then begin to progress involving the skin, joints, nervous system and heart

(Franz and Krause 2003). These symptoms can be ambiguous and mistaken for the common cold, flu, or a viral infection, and thus Lyme disease often goes untreated and undiagnosed. Although erythema migrans is the main clinical identifier of Lyme, not all tick bites lead to the bullseye rash, with only 80% resulting in erythrema migrans, according to the CDC (Allen et al. 2016) If erythema migrans is not present, Lyme disease is only diagnosed through a combination of serology tests taken within a close time frame of being bitten, although antibodies against the spirochete are often not produced in the early stages of infection.  Further relevant information for clinicians properly diagnosing a Lyme infection includes being bitten in an area known to be endemic for Lyme disease, and having symptoms compatible with Lyme disease (Franz and Krause 2003).

*Ixodes scapularis* has four life stages – egg, larval, nympal, and adult stages. A female adult will engorge a blood meal and lay eggs in a single gonotropic cycle. Once hatched, larva will feed on birds or small mammals such as mice, and then moult into nymphs. Nymphs will also feed on small mammals, while adults will feed on medium to large mammals such as deer (Ribeiro et al. 2006). *I. scapularis* first must find a suitable host to feed on, attach to the host, and then engage in blood meal engorgement. Transmission of *B. burgdorferi* occurs during tick-host attachment when the tick is engorging on a blood meal. It is through the salivary gland excretion of saliva back into the host integument that allows for the transmission of *B. burgdorferi*.  *B. burgdorferi* is known to be transmitted to mammals through immature nymphs and adult female *I. scapularis* ticks, as adult males do not feed long enough for the spirochete to be transferred through the excreted saliva (Falco et al. 1999).

*I. scapularis* was first found in northwestern Wisconsin in the late 1960s, and since then has expanded both southward and eastward (Guerra 2002).  In the 1990s, only 1 population of *I.*

*scapularis* was found in Canada, along the shore of Lake Erie. Since then, populations have been reported throughout Ontario, Nova Scotia, New Brunswick, and southeastern Manitoba, with potential emerging populations in southern Quebec (Dphil et al. 2009; Gasmi et al. 2017). The emergence and expansion of *I. scapularis* in Canada has been suggested to be due to sudden warmer climates (Dphil et al. 2009). The widespread increase of *I. scapularis* in Canada has made way for the subsequent increase of reported cases of Lyme disease.

**Salivary gland morphology**

Ixodid tick salivary glands are important organs which contribute to the survival of ticks. The salivary glands are involved in the transmission of pathogens, attachment to the host integument, water absorption from air during off-host periods, and are also involved in the excretion of excess ions and fluids during feeding periods (Sauer et al. 1995). The salivary glands are involved in secreting a saliva which contains cement that allows the tick to attach to the host integument. This saliva is composed of a variety of enzymes and inhibitors, histamine, prostaglandins, and immunomodulatory factors which help suppress the host immune response, allowing the tick to attach and engorge a blood meal successfully (Sauer et al. 1995). Ixodid tick salivary glands are composed of three acini types in females (I-III), and four acini types in males (I-IV) (Sauer et al. 1995). The salivary glands are located on both sides of the tick body, lying anterolaterally and extending posteriorly (Figure 1). There is a main salivary duct from each salivary gland that enters a tube known as the salivarium, which opens into the food channel (Sauer *et al.* 2000). Attached to the anterior region of the main duct are the agranular type I acini, which are void of secretory granules. Type I acini contain four cell types, which include a lamellate central cell, pyramidal cells, peritubular cells, and constrictor cells. Type II acini are granular, containing secretory granules in the cytoplasm of the cells (Sauer *et al*. 2000). Type II

acini contain six types of cells (a,b, and $c_1$-$c_4$), and are known to change morphologically during and after tick feeding (Sauer et al. 1995). Type III acini are granular and made up of three cell types (d-f), and two additional interstitial cells known as adlumenal, which are towards the lumen of the acini, and ablumenal, which are away from the lumen of the acini (Sauer *et al*. 2000).

Type III acini are the most abundant in the salivary glands and are found on the peripheral and posterior regions of the salivary glands on both sides of the organism. During tick feeding, the nuclei and cytoplasm of the cells within type III acini enlarge, causing an increase in the mass of the acinus without an increase in the number of cells (Sauer *et al.* 2000). The f cells in type III acini undergo cell transformation in which the plasma membranes and mitochondria multiply along with an increase in ablumenal interstitial cell mitochondria and plasma membrane. These together form a basal labyrinth which is common to fluid transporting epithelia, and thus it is believed that during tick feeding, fluid is transported through type III acini. In unfed ticks, the type f cells are agranular, however become granular and increase in size during feeding. Type IV acini are only present in male ticks, and contain adluminal, abluminal interstitial cells, and a type g cell which fills with secretory granules during feeding.

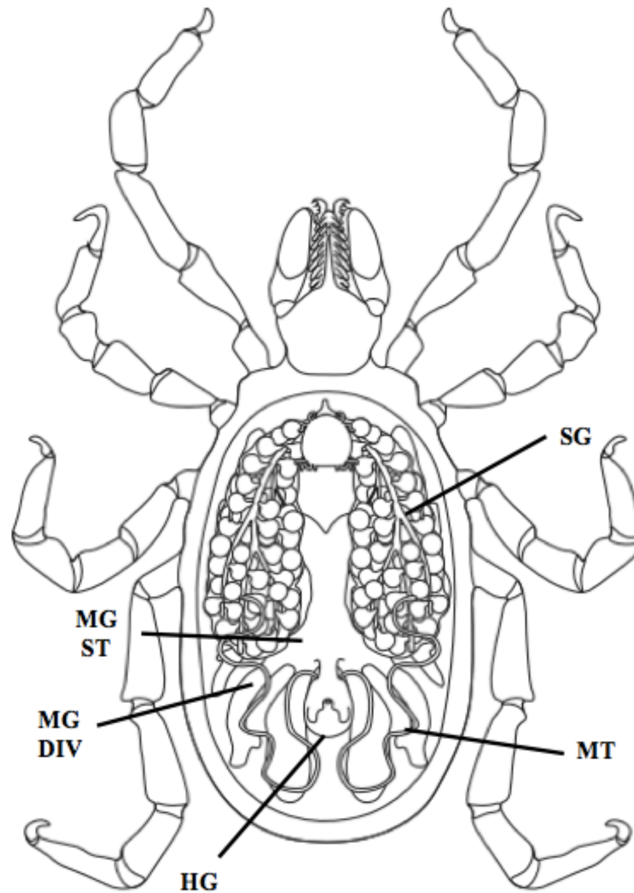


**Figure 1**. Schematic of the internal anatomy of adult *Ixodes scapularis*, the black-legged tick. The salivary glands (SG) are paired structures located on both sides of the organism extending posteriorly and lying anterolaterally. Posterior to the salivary gland, located in the middle of the body, lies the midgut which contains the central portion, the stomach (ST) and several midgut diverticula (DIV) extending from it. Posterior and ventral to the midgut is the hindgut (HG) composed of the rectal sac and intestine. Not seen is the intestine, which extends ventral and posteriorly from the midgut to form a junction with the rectal sac. At this junction exit two Malpighian tubules (MT). Illustration was created by Lesia Scyza.

## Ion and osmoregulation in *Ixodes scapularis* salivary glands

*Ixodes scapularis* are obligate blood-feeding ectoparasites that must regulate ion and fluid levels due to their hematophagous feeding strategy. During on-host feeding, *I. scapularis* will stay attached to the host integument from a few days for up to several weeks, and thus must regulate fluid levels in order to generate sufficient saliva for the lengthy feeding period (Bontemps-Gallo, Lawrence, and Gherardini 2016). *Amblyomma americanum,* an Ixodid tick, attaches to a host and feeds for 7 to 14 days, with a period of rapid feeding lasting between 24 and 48 hours. During this feeding bout, the adult female's body weight increases by up to 100 times its unfed weight with excess fluid being excreted back to the host through the salivary glands (Sauer, Essenberg, and Bowman 2000). Water is recycled from the blood of the host, through the haemolymph of the tick to the salivary glands where it is excreted back to the host through the salivary secretions. This flux of water occurs concurrently with transport of $Na^+$, $K^+$, and $Ca^{2+}$ ions (Figure 2). During blood feeding, the tick excretes 70% of the excess fluids back into the host through secretions by the salivary glands, and thus the salivary glands are recognized as being the main on-host iono- and osmoregulatory tissues within Ixodid ticks (Bontemps-Gallo, Lawrence, and Gherardini 2016).

The salivary glands are critical organs to tick survival as they are what allows for this obligate hematophagous feeding strategy in Ixodes, and additionally, are of significant medical importance as well, as they allow for pathogen transmission. Due to their multifunctional and differing roles, the salivary gland acini have been characterized into three distinct types – each contributing to different physiological properties within the organism. Type I acini are recognized for their role in ion reabsorption and uptake of fluid from the atmosphere. Type II acini are characterized as being involved in the secretion of saliva that contains several immune-

suppressing proteins which allow for the inhibition of the host immune response. Lastly, type III acini are recognized for their role in ridding the organism of excess ions and fluids, which are taken up during the blood meal, playing a critical role to their survival. The mechanisms responsible for and which contribute to the multi-functionality of the acini types have been described below; however, are still being studied and characterized to date.

The salivary glands are innervated, and fluid secretion is controlled by neurochemicals including dopamine and neuropeptides MIP (myoinhibitory peptide) and SIFamide through innervation (Šimo, Žitňan, and Park. 2009). MIP and SIFamide are colocalized in protocerebral cells, with their projections innervating the salivary gland, however their specific role in regulation has not yet been elucidated  Salivary gland secretion is also controlled through locally synthesized dopamine as a paracrine factor (Koči et al. 2014; Kim, Šimo, and Park 2014) On a physiological level, two actions occur in type III acini allowing for osmoregulation regulated by dopamine; inward fluid transport, and the release of the luminal content within the acini.

Studies have found that dopamine induces salivary gland secretion through extracellular $Ca^{2+}$ and cAMP (Kim, Šimo, and Park 2014). Recent work has characterized two dopamine receptors, specifically dopamine receptor D1 and invertebrate specific D1-like (InvD1L) dopamine receptor, in type II and III acini (Kim, Šimo, and Park 2014). The D1 receptor is found in the epithelial cells of acini while the InvD1L receptor is found in axonal projections that reach the basal area of the acini, and in another axonal projection that extends to the apical region of acini (Kim, Šimo, and Park 2014).  The D1 receptor is coupled to an increase in cAMP in epithelial cells, while the InvD1L receptor is coupled to $Ca^{2+}$ mobilization in the myoepithelial cells of type II and III acini.  (Kim, Šimo, and Park 2014). Low dopamine doses are found to activate D1 receptors causing cAMP-mediated fluid transport, while high dopamine doses act on

the InvD1L receptor to induce $Ca^{2+}$-mediated myoepithelial cell contraction allowing for saliva efflux from the salivary glands. Thus, dopamine acts on D1 receptors for water-solute transport into the acinar lumen while acting on the InvD1L receptors for pumping/gating within the type III acini (Kim, Šimo, and Park 2014). Dopamine acts through these two receptors to allow for the influx of water and solutes in type II and III acini and also allows myoepithelial cells in the acini to contract (Kim et al. 2016).

$Na^{+}/K^{+}$-ATPase (NKA) is an enzyme solute pump which allows for the movement of sodium out of cells simultaneously with the influx of potassium into the cells. NKA is known to play a role in salivary gland secretion in Ixodid ticks as the application of ouabain, a known NKA inhibitor, decreases dopamine induced salivary secretion at low concentrations, and completely abolishes it at high concentrations (Kim et al. 2016). It is important to note that Type I acini have been described as being involved in absorption of water vapor from the atmosphere through the use of a hygroscopic saliva during off-host periods, while the role of type II and III acini include the secretion of saliva that is rich in bioactive proteins to suppress the host immune response and excretion of excess ions and fluids during on host feeding. NKA inhibition was examined in type I and III acini and was found to have differing levels of inhibition for NKA. A 1 μmol $l^{-1}$ ouabain treatment was found to inhibit the resorption function of type I acini as saliva secreted was hyperosmotic, while only lowering the secretory function of type III acini. At a high dose of 10 μmol $l^{-1}$ both functions were inhibited. As the high dose was able to abolish salivary gland secretion, NKA is suggested to play a major role in the downstream mechanism induced by dopamine. NKA is located within the infoldings of the apical membranes of epithelial cells in the type III acini, while in the type I acini, it localizes to the basolateral surface. NKA is known to be located in basolateral membranes in other insects; however, in the salivary

glands of the American cockroach, NKA is found apically in peripheral acinar cells that function to produce saliva, while it is located basolaterally in duct cells where its function is for sodium resorption (Just et al. 1996). These findings suggest that NKA serves two roles within the salivary glands of the tick; ion and water influx in the type III f cells lumen, which allows for the formation of saliva, and ion reabsorption in type I acini. Ion reabsorption occurring in type I acini was later confirmed with experiments examining Rhodamine123, a tracer dye used to examine membrane transport. Dehydrated ticks were fed with water containing this dye, in order to examine the route of water absorption which was found solely to be within type I acini cells in unfed ticks.

In summary, type III acini are involved in ion and fluid regulation during on-host feeding, through the excretion of a saliva back into the host which carries excess ions and fluids. During feeding, f cells within the type III acini undergo cell transformation where the plasma membranes and mitochondria of the albumen interstitial cells multiply, allowing for the formation of a basal labyrinth which is a marker for fluid transportation occurring within epithelial cells (Šimo, Koči, and Park 2013) . The two dopamine receptor types the D1 receptor and InvD1L receptor are coupled to an increase in cAMP within epithelial cells and calcium mobilization in myoepithelial cells of type II and III acini, respectively (Kim, Šimo, and Park 2014). Dopamine is known to be a primary inducer of salivary gland secretion, acting through these receptors during feeding periods to allow for both water-solute influx into the acini, and pumping/gating. NKA inhibition using oubain at high and low doses was found to terminate salivary gland secretion, and cause hyperosmotic saliva to be secreted, respectively. This, along with the identification of a water absorption pathway within type I acini, suggests that type I acini, in contrast to type III acini, are involved in ion reabsorption although the underlying

mechanisms are not fully known. Although not fully characterized, the type I acini are involved in the tick producing a hygroscopic saliva that makes its way to the surface of the hypostome, near the mouth area, to allow for the absorption of atmospheric moisture, allowing the tick to stay hydrated during off-host periods (Francischetti et al. 2009).

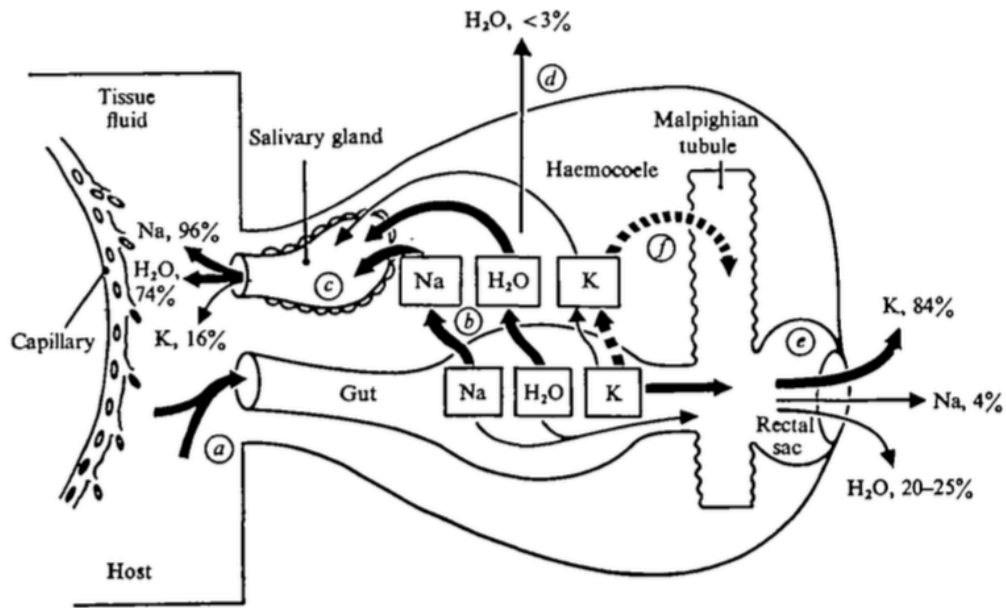


**Figure 2**. The influx and efflux of ions and fluid by a female tick during on-host feeding. The salivary glands are suggested to account for 70% of fluid excreted back into the host. The Malpighian tubules are suggested to be involved in potassium excretion. Excess ions are said to be taken up in the blood meal and travel from the gut to the salivary glands where they are excreted back into the host. Potassium ions enter the Malpighian tubules where they are suggested to be excreted as nitrogenous waste. Thick arrows suggest major routes while fine arrows suggest minor routes. Dashed arrows suggest possible routes of potassium excretion. Percentages refer to proportions of total amount of ions excreted during the feeding period (Kaufman and Phillips 1973).

**Insect ion and osmoregulation**

The salivary glands are currently recognized as being the main iono and osmoregulatory tissues in the black-legged tick, *I. scapularis*. Considering that the salivary glands only account for the excretion of 70% of excess ions and fluids back into the host during blood feeding, another mechanism must be considered for complete iono- and osmoregulation. In other hematophagous arthropods, such as the insects *Rhodnius prolixus,* and *Aedes aegypti*, the main iono-and osmoregulatory tissues are the Malpighian tubules. *Aedes aegypti* are of focus in the lab, and being that they too are blood feeding disease vectors, albeit with a more fully characterized excretory system, they will be discussed in further detail. *Aedes aegypti* are vectors for a variety of arboviruses with a global distribution (Kraemer et al. 2015). The pathogens transmitted by *A. aegypti* include the causative agents leading to human diseases such as yellow fever, dengue, and chikunguya. Dengue virus is distributed throughout the tropics, as well as Europe, and is considered the most common arbovirus, with the potential to infect 100 million people globally, leaving half the global population at risk for possible infection (Kraemer et al. 2015). Chikungunya has recently appeared in America and also has resulted in recent outbreaks in Italy (2007) and France (2010, 2014). The distribution and appearance of such diseases has increased with little progress on vaccines or methods of prevention to date, and thus the only method to prevent increase in disease incidence amongst humans, is to control and limit the spread of *A. aegypti* as a disease vector (Piermarini *et al* 2017).

The Malpighian tubules of mosquitos are the main iono and osmoregulatory tissues in that they play a role in both excreting excess ions and fluids after a blood meal, and are also involved in excreting nitrogenous waste from the digestion and metabolism of blood (Piermarini, Esquivel, and Denton 2017). *A. aegypti* has five Malpighian tubules which combine

with the ileum and rectum to form the renal system. The Malpighian tubules are made up of two types of cells; principal and stellate. Principal cells have an apical luminal brush border which fold to form the lumen of the tubule, while stellate cells have a infolded basolateral membrane (Piermarini, Esquivel, and Denton 2017). Nitrogenous waste excretion by the Malpighian tubules occurs by transepithelial fluid secretion. $Na^{+}$, $K^{+}$, and $Cl^{-}$ are transported from the haemolymph across the tubule lumen by epithelial cells allowing for the generation of an osmotic gradient allowing fluid to follow. This allows for an isosmotic urine which is then modified by the hindgut before final excretion from the mosquito (Piermarini, Esquivel, and Denton 2017). Secondary transport of ions and fluids from the haemolymph to the tubule lumen occurs via an electrochemical gradient generated by a V-type $H^{+}$-ATPase (VA) in the apical brush border which pumps protons from the cytosol into the tubule lumen, creating a negative membrane voltage across both apical and basolateral membranes (Figure 3). This in turn allows for the uptake of ions from the haemolymph across the basolateral membrane through various ion channels such as a $Na^{+}$ channel (Piermarini, Esquivel, and Denton 2017). Other suggested channels, such as Kir1 and Kir2B, will not be discussed here. Within both basolateral and apical membranes, there exists proposed transporters and exchangers such as cation chloride co-transporters such as $Na^{+,}$ $K^{+}$, $Cl^{-}$ co-transporter, KCl co-transporter, Na/H exchangers, and anion exchangers such as $HCO^{3-}$ or $Cl^{-}$ (Figure 3). These allow for the movement of NaCl and KCl from the haemolymph to tubule lumen (Figure 3). The movement of ions and fluids from the haemolymph to the tubule lumen in response to a blood meal allows for the excretion of a urine that rids the mosquito of 40% fluids and $Na^{+}$, 145% of $K^{+}$, and 60% of $Cl^{-}$. *A. aegypti*, like *I. scapularis*, is a blood feeding arthropod facing similar diet-associated challenges to overall homeostasis and is also a prominent disease vector, and thus it is of value to consider knowledge

of the main ionoregulatory and osmoregulatory mechanisms in this organism, to better understand mechanisms occurring in *I. scapularis.*

**Second messengers and insect ion and osmoregulation**

In insects, cyclic GMP and cyclic AMP act as second messengers for various endocrine factors potentially inhibiting or stimulating tubule secretion rates. The neurohormone serotonin stimulates tubule secretion *in vivo* in *R. prolixus,* acting through cAMP as a second messenger (Quinlan *et al*. 1997). In contrast, CAP2B, a cardioactive peptide (that belongs to a family of neuropeptides more recently referred to as CAPA peptides or periviscerokinins, PVK) decreases secretion rates of serotonin stimulated Malpighian tubules through cGMP-mediated pathways, thus promoting anti-diuresis (Quinlan *et al*. 1997). In *Tenebrio moliter,* the mealworm, corticotropin-releasing factor (CRF-related) diuretic peptides stimulate tubules *in vivo* via cAMP, while anti-diuretic peptides inhibit secretion via cGMP (Wiehart et al. 2002). The CAPA peptide *Aedes* Capa-PVK-1 promotes diuresis in larval *A. aegypti* when applied at high doses via cGMP mediated activation of PKA (Ionescu and Donini 2012). Low doses of Capa-PVK-1 promote anti-diuresis via NOs/cGMP/PKG pathways (Ionescu and Donini 2012). However, in *R. prolixus,* Capa-PVK acts as an anti-diuretic peptide. In the salivary glands of *I. scapularis*, dopamine is known to stimulate secretion through the activation of cAMP pathways (Kim *et al*. 2014). The role of second messengers in regulating ion and osmoregulation in organs is important for the characterization of the excretory system in *I. scapularis*.

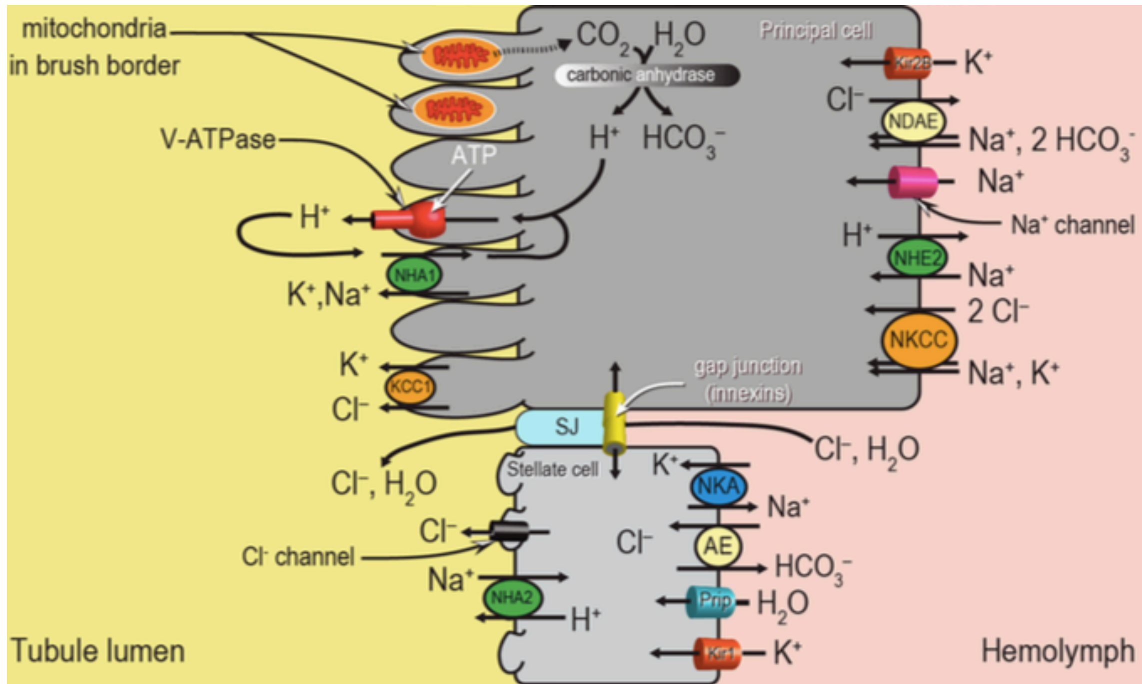


**Figure 3.** Model of fluid secretion in *Aedes aegypti* Malpighian tubules. A proton pump located at the apical brush border of principal cells allows for the subsequent movement of ions from the haemolymph across the cells to the tubule lumen. At basolateral and apical membranes, there are proposed cation chloride co-transporters such as the $Na^{+,}$ $K^+$, $Cl^-$ cotransporter, K-Cl co-transporter, as well as Na/H exchangers, and anion exchangers such as $HCO^{3-}$ or $Cl^-$. The movement of ions across these cells allows for the excretion of urine post blood meal. Figure originally from Piermarini et al., 2017.

## *Ixodes scapularis* midgut and digestion

The midgut is made up of two parts: a stomach (central ventriculus) and various diverticula which extend from the stomach; both which contribute to the processing of the blood meal (Sonenshine and Roe 2014). The midgut is surrounded by a layer of smooth muscle and an epithelium which contains two cell types, including undifferentiated cells and digestive cells. When a tick is taking up a blood meal, the digestive cells develop from undifferentiated cells for the ingestion of haemoglobin (Lara et al 2005). The distal portion of the digestive cells fill with haematin, break off, and are transported through the gut to the rectal sac for excretion of nitrogenous waste (Sonenshine and Roe 2014). The morphological changes that occur in the midgut during feeding mirror the four feeding stages: attachment, slow feeding, rapid feeding, and detachment for blood meal processing. During the attachment phase, in which the tick is attaching to the host, the midgut is composed mainly of undifferentiated cells (Coons et al. 1986; Sonenshine and Roe 2014). The next phase, the slow feeding phase, lasts up to 5 days and undifferentiated cells begin to differentiate into digestive cells, which will serve to digest the blood meal. In the next phase, the rapid feeding phase, the gut is mainly composed of digestive cells, which increases until the final stage of detachment in which the blood meal is fully digested (Sonenshine and Roe 2014). Digested food and waste is then moved to the Malpighian tubules and rectal sac via the intestine.

### *Ixodes scapularis* Malpighian tubules and hindgut

Metabolic wastes are excreted from the tick through the actions of organs in the tick hindgut, which is made up of the intestine, rectal sac, anal canal, and Malpighian tubules. In other blood feeding arthropods, such as *A. aegypti,* the hindgut is made up of an ileum, rectum, 5 Malpighian tubules, and anus. In *I. scapularis*, the hindgut begins with the intestine, which is a short narrow tube lined with a single layer of epithelial cells, which can be cuboidal or columnar. The intestine comes off the main portion of the midgut postero-ventrally and forms a junction with the rectal sac (Sonenshine and Roe 2014). When the intestine is filled with fluid, it is easily mistaken for a midgut diverticula branching off the main portion of the midgut. The intestine is surrounded by a layer of circular and longitudinal smooth muscle cells, allowing for its contraction which permits the passage of haematin from the midgut to the rectal sac, and nitrogenous waste from the Malpighian tubules to the rectal sac through the intestine (Sonenshine and Roe 2014). Forming a junction with the intestine is the rectal sac, which lies in the midline of the body both ventrally and posteriorly. The rectal sac is lined with a layer of epithelial cells which can be cuboidal or squamous and is surrounded by a thin layer of circular musculature, which in contrast to insects, is not lined with cuticle (Sonenshine and Roe 2014). The rectal sac usually appears as white and full due to the nitrogenous waste, guanine crystals, accumulating within the rectal sac (Dantas-Torres 2010). At the junction of the intestine and rectal sac are where the Malpighian tubules enter. The rectal sac is suggested to be involved in osmoregulation through the concentration of urine from the Malpighian tubules (Campbell et al. 2010). The Malpighian tubules are involved in excretion of nitrogenous waste as guanine crystals. *Ixodes scapularis* contain a pair of Malpighian tubules which extend from the intestine-rectal sac junction and loop around the body organs. The Malpighian tubules consist of cuboidal

cells surrounded by smooth muscle. The Malpighian tubules also contain cells which have a brush border layer of microvilli, in addition to cells with the basal labyrinth on the outside facing the haemolymph (i.e. basolateral surface), characteristics that are analogous to insect Malpighian tubule cells, which allow for fluid transport (Sonenshine and Roe 2014). Lastly, in the most posterior region of the rectal sac lies the anal canal, which connects the rectal sac with the anal aperature and contains a layer of epithelial cells and smooth muscle which function in waste defecation.

***Ixodes scapularis*** **excretion and waste elimination**

Unlike many insects, such as *A. aegypti,* the mechanisms and biochemistry of excretory processes occurring in the tick Malpighian tubules and rectal sac are not completely characterized. It has been suggested that ion resorption occurs in the proximal area of the Malpighian tubules; however, this has not yet been described (Sonenshine and Roe 2014). The rectal sac is suggested to be involved in osmoregulation through the concentration of urine from the Malpighian tubules. This is believed to occur via an aquaporin, IrAQP1, which was found to be expressed and associated with organs containing high water flux, such as the salivary glands and hindgut (Campbell et al. 2010). Aquaporins are molecules which allow for transmembrane water transport via formation of pores in the cell membrane (Sonenshine and Roe 2014). Different species of ticks have been found to express aquaporins within the rectal sac and Malpighian tubules, such as *Rhipicephalus sanguineus*, the brown dog tick, in which the aquaporin RsAQP1, was found in the Malpighian tubules and rectal sac (Ball et al. 2009). The luminal lining of epithelial cells within the rectal sac also were found to have multiple microvilli which form a brush border, analogous to cells involved in water passage. Unlike insects, which excrete nitrogenous waste primarily as uric acid, the preservation of body water by Ixodid ticks

is made possible through the elimination of nitrogenous wastes from their body through the excretion of guanine crystals, which are formed in the Malpighian tubules (Dantas-Torres 2010). Guanine crystal excretion is advantageous in comparison to uric acid excretion by insects as it allows for the conservation of body water. More research is required in characterizing the role of the Malpighian tubules and hindgut in waste excretion in order to fully understand their roles in whole body ion and water regulation in Ixodid ticks.

**Objectives and Hypotheses**

Ticks are obligate blood feeding ectoparasites with the potential to transmit a variety of infectious pathogens including rickettsiae, spirochetes and protozoa (Horn et al. 2009). The salivary glands are responsible for excreting back about 70% of ions and fluids back to the host during blood feeding (Sauer et al. 1995). Due to the roles they play in pathogen transmission and hydromineral balance, the tick's salivary glands are considered the most vital organ, and subsequently, a large portion of research and attention has been focused on them. However, the role of other tissues and organs in regulating ion and fluid balance, such as the midgut, hindgut and Malpighian tubules, must be considered, as homologous structures are known to be involved in iono and osmoregulatory processes in other blood feeding arthropods such as *A. aegypti.*

The objective of this research was to further characterize organs in the black-legged tick, *I. scapularis*. Specifically, the goal was to characterize potential organs involved in ion and osmoregulatory processes, such as the salivary glands, midgut, intestine, rectal sac, and Malpighian tubules. *I hypothesized that NKA and VA distribution would differ in the salivary glands, midgut, hindgut, and Malpighian tubules as each organ would presumably play a different role in ion and osmoregulation after blood meal engorgement.*

So far, much of the literature on tick salivary glands has largely focused on pathogen transmission, and research on the mechanisms underlying ion and osmoregulation within the salivary glands both during and after feeding are fairly novel. Recent research found that NKA has two differing roles within the salivary gland acini. In type III acini, it is suggested to function in the production of a hyperosmotic saliva, while in type I acini, it is suggested to allow for ion resorption as the distribution of NKA differs between these two acini (Kim et al 2016). My goal was to specifically further characterize the distribution of NKA within the salivary gland acini, and to also examine the distribution of VA as this ATPase is known to generate the gradient which allows for the passage of ions between the haemolymph and tubule lumen in Malpighian tubules of insects. Specifically, I expected that NKA and VA distribution within the three salivary gland acini types would differ, as each acini type plays a differential role within the tick. NKA has been found to be located apically in the type III acini, while located basolaterally in the type I acini (Kim et al. 2016), which differs from insects in that NKA is usually located in the basolateral membrane (Patrick et al. 2006). Furthermore, VA activity within the salivary glands has scarcely been looked at and thus the aim of this research was to characterize its distribution within the differing acini types. Given that type I acini are involved in ion and osmoregulation during off-host periods, while type III are involved during on-host feeding, I predict I will find different intensities of immunoreactivity of these ATPases. Specifically, I expected to find high activity levels of VA and NKA within the salivary glands due to their main role in ion and osmoregulation. This was reinforced due to the fact that NKA and VA allow for fluid and ion transport across epithelia, specifically in ion and osmoregulatory organs, as seen in *A. aegypti* (Piermarini, Esquivel, and Denton 2017).

My second goal was to characterize the midgut and hindgut, specifically the rectal sac and intestine in the hindgut, in determining the distribution of NKA and VA within these gut organs. Research on characterizing the potential role of ion and osmoregulation in these organs is fairly novel and thus my aim was to characterize NKA and VA distribution across these organs using methods involving immunohistochemistry and enzyme activity assays, which can determine the localization and measure activity levels of NKA and VA. In the midgut and hindgut, I expected that the distribution of NKA and VA will differ across the two cell types in the midgut. Moreover, I expected that the distribution NKA and VA would differ between the intestine and rectal sac. As mentioned earlier, the midgut contains two cell types; digestive cells and undifferentiated cells, depending on the stage of blood meal digestion (Sonenshine and Roe 2014). The midgut diverticula and stomach have different roles in aiding blood digestion, and thus it is expected NKA and VA localization would be different between the two tissues. The rectal sac in ticks allows for the excretion of nitrogenous waste, and it is suggested that the rectal sac allows for the reabsorption of ions and fluids before final excretion, however this has yet to be examined. In Ixodes*,* aquaporins have been found throughout the rectal sac which is suggestive of the potential involvement of the rectal sac in ion and fluid reabsorption. Thus, I aim to characterize the possibility of ion reabsorption in the rectal sac through the examination of NKA and VA activity and their distribution using immunohistochemical methods. The intestine is what allows for the passage of waste into the rectal sac, and it is presumed there would be minimal NKA and VA activity found here.

Lastly, my aim was to examine the potential role of Malpighian tubules in ion and osmoregulation in *I. scapularis* through determining whether second messengers known to influence insect Malpighian tubules, such as cAMP and cGMP, are active in the Malpighian

tubules of *I. scapularis*. The Malpighian tubules of several blood feeding arthropods such as *A. aegypti* and *Rhodnius prolixus* are known to be the main iono and osmoregulatory tissues within these organisms. However, as previously mentioned, much of the research on ticks has been dedicated to determining the mechanisms underlying and preventing pathogen transmission. There is minimal research on the Malpighian tubules of ticks, and my goal was to provide a baseline characterization of these highly-understudied organs in ticks. My first aim was to examine NKA and VA distribution through the analysis of immunohistochemistry. My second aim was to determine NKA and VA activity levels through the activity assay. I expected NKA and VA to be distributed along the Malpighian tubule length, with NKA located basolaterally and VA apically, as similar differential localization was found in the Malpighian tubules of another blood-feeding arthropod, *Aedes aegypti* (Patrick et al., 2006). The Malpighian tubules of *A. aegypti* are known to have NKA and VA distribution, with a basal NKA and apical VA allowing for the influx of ions and fluids across the tubule cells from the haemolymph to the lumen for excretion (Piermarini, Esquivel, and Denton 2017). Cyclic AMP and cyclic GMP act as second messengers to a variety of endocrine factors which possess stimulatory and inhibitory effects on Malpighian tubule secretion in insects such as *Aedes aegypti* and *Rhodnius prolixus*.

Due to their central role in regulating the activity of other hematophagous insect Malpighian tubules, *I hypothesized that cAMP and cGMP would influence I. scapularis Malpighian tubule secretion rates in vitro*. I examined possible Malpighian tubule secretion stimulated by 8-bromo cAMP and 8-bromo cGMP through the procedure of Ramsay assays, which involve the isolation of Malpighian tubules in a droplet of saline similar to the haemolymph of the organism. The distal portion remains in the droplet while the proximal portion is attached to a pin outside the droplet. Secretion rates and the effect on secretion by

several inhibitors or stimulators can then be observed over a desired time (Ramsay, 1954).

Lastly, I also examined NKA and VA activity quantitatively through an enzymatic activity assay. Given its proposed role in reabsorption, *I hypothesized that the hindgut – made up of the intestine, rectal sac, and Malpighian tubules, will have high NKA and VA activity levels*. *I also hypothesized that the salivary glands, which account for the majority of ions and fluids excreted back to the host during on host feeding, would have high NKA and VA activity levels.*

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# CHAPTER II:

# DISTRIBUTION OF IONOMOTIVE PUMPS AND CONTROL OF MALPIGHIAN TUBULE SECRETION IN THE BLACK-LEGGED TICK, *IXODES SCAPULARIS*

**Abstract**

Osmoregulation and ion regulation in ticks are pivotal to their survival as their haematophagous feeding strategy results in the presence of excess ions and fluid during blood meals. The salivary glands are known to be essential tissues in Ixodid ticks as they play a role both in absorption of water vapor from unsaturated air, and pathogen transmission. The salivary glands are also essential for tick hydromineral balance as they play a vital role in excretion of excess ions and fluids back to the host during blood meal engorgement. The Malpighian tubules of ticks are also suggested to play a role in this regulation since the salivary glands only account for 70% of water excretion back into the host. Apart from the salivary glands, the involvement or role of other organs in ion and water regulation is not known. This study aimed to characterize organs potentially involved in ion and osmoregulation by examining the distribution and activity of $Na^+/K^+$ ATPase and V-type $H^+$ ATPase, as these are both suggested to drive ion transport across epithelial cells. $Na^+/K^+$ ATPase and V-type $H^+$ ATPase immunoreactivity was localized to the midgut, salivary glands, Malpighian tubules, intestine and rectal sac. Secretory activity of the Malpighian tubules was also examined using the Ramsay assay, which determined that the second messengers cAMP and cGMP both stimulate fluid secretion. Given that *I. scapularis* are blood feeding disease vectors, ion and osmoregulation are crucial to their survival and thus further understanding all of the tissues and mechanisms underlying these processes are critical in potentially developing novel mechanisms for the control of tick vectors and thus a reduction in tick-borne diseases.

**Introduction**

Ticks are obligate blood feeding arthropods and carry a great variety of pathogenic organisms which include bacteria, viruses, protozoa, helminth parasites, and even pathogenic nematodes (Hughes 2005). There is a large range of human diseases caused by ticks such as Rocky Mountain spotted fever, human granulocytic anaplasmosis, human monocytic anaplasmosis, tularemia, Colorado tick fever, and tick-borne encephalitis (Ribeiro et al. 2006). Ticks are classified into three families; Argasidae, also known as soft ticks, Nuttalliellidae, consisting of only a single species, and Ixodidae, or hard ticks, to which this chapter will be focused on. Ixodidae are the most prominent tick family, with the largest number of species and are also the family with the greatest potential to be vectors of disease (Oliver 2017). Ixodidae have a four stage developmental cycle as eggs, larva, nymph and adults, emerging to the next stage after a blood meal is acquired by post-embryonic stages (Oliver 2017). This chapter specifically focuses on the blacklegged tick, *Ixodes scapularis*, a species of ticks from the Ixodidae family which are commonly known as principal vectors for Lyme disease in North America (Guerra 2002). Cases of Lyme disease have risen from 144 to 917 since 2009 (Levy 2014; Gasmi et al. 2017). In Ontario, the most abundant tick species is *I. scapularis*, which the highest abundance in Eastern Ontario (Nelder et al. 2014). Due to their role as chief vectors of the spirochete of *Borrelia burgdorferi,* the abundance of *I. scapularis* in North America poses implications for the spread of Lyme disease. The engorgement of a blood meal is critical to the survival of *I. scapularis* as it is necessary for successfully moulting to the next life stage. The feeding cycle includes finding a host, attaching to it, and taking up the blood meal. It is during the attachment stage in which the tick excretes a saliva containing bioactive proteins which prevent the host immune response through the prevention of coagulation, blocking haemostasis

using anti-haemostatic factors, and preventing vasoconstriction by the host (Bowman et al. 1997). It is in this saliva that the spirochete *B. burgdorferi* is transmitted to the human host. Prior to transmission during a blood meal, the spirochete lives in the tick midgut until the commencement of the blood meal, in which it travels to the salivary glands through the haemolymph and multiplies for transmission (Pal et al. 2004). Once the *B. burgdorferi* spirochete is transmitted to a human host, Lyme disease occurs in stages in which different clinical manifestations appear in the skin, nervous system, cardiac system, musculoskeletal and digestive system. Little is known regarding the physiology of *I. scapularis,* and in order to develop strategies that can potentially control tick population and the threats they pose via pathogen transmission, it is important to understand their basic physiology given that blood meal engorgement is crucial to their survival, and relatedly, mechanisms of ion and osmoregulation.

The salivary glands are involved in many functions attributing to the physiology and survival of *I. scapularis*. The salivary glands facilitate pathogen transmission, absorption of water vapor from unsaturated air, and more importantly, ion and osmoregulation during host feeding (Sonenshine 2014). In *I. scapularis*, the salivary glands are of an alveolar structure composed of both agranular and granular acini (Sonenshine 2014**)**. The agranular acini play a vital role in osmoregulation while the granular acini are involved in the secretion of bioactive proteins and lipids (Sonenshine 2014). Type I acini are suggested to be involved in tick water balance due to their highly convoluted membrane infoldings, which extend from the outer cell membrane enclosing several vacuoles and mitochondria that allow for active transport and fluid regulation (Needham et al 1990). Type I acini are believed to facilitate the absorption of water vapor from the atmosphere through the secretion of a hygroscopic saliva, which in turn prevents *I. scapularis* from dehydrating during off-host periods (Kim et al. 2016). Type II and Type III

acini are granular, and contain cell types mainly involved in production of cement which allows for host attachment. They are also involved in inhibiting the immune response of the host, by blocking hemostasis using anti-hemostatic factors, allowing for the prevention of platelet adhesion, blood coagulation, and vasoconstriction by the host (Bowman *et al.* 1997). The type III acini are mainly recognized for their function in excretion of excess ions and fluids back to the host during the blood meal, which allows for ionic and osmotic homeostasis (Kim et al. 2016). *I. scapularis* are faced with the challenge of regulating both ion and fluid levels as they engorge during host feeding. While the tick is attached and feeding on the host, they are able to concentrate their blood meal and secrete any excess fluid or ions they do not require back to the host through excretion of saliva via their salivary glands (Bowman et al. 1997). This in turn, allows for the prevention of the uptake of excess ions or fluids. As the salivary glands account for the majority of the excess ions and fluids excreted back to the host, they are considered critical to the survival of the organism by ensuring ion and osmoregulation, and thus are considered the main iono and osmoregulatory tissues of the organism.

*I. scapularis* differ from other blood feeding arthropods given that they are able to excrete excess ions and fluids back to the host simultaneously while taking up a blood meal. *Aedes aegypti* also face the challenge of ingesting an excess of ions and water during a blood meal that must be removed rapidly to ensure survival. While engorging, *A. aegypti* maintain ion and osmotic homeostasis through the Malpighian tubules, which begin diuresis concurrently while blood meal engorgement occurs (Tiburcy, Beyenbach, and Wieczorek 2013). The Malpighian tubules of *A. aegypti* are composed of principal cells, which have an apical brush border which folds to form the lumen of the tubule, while stellate cells have an infolded basolateral membrane (Piermarini, Esquivel, and Denton 2017). During diuresis, nitrogenous

wastes are excreted through the transepithelial fluid secretion of ions. $Na^+$, $K^+$, and $Cl^-$, are transported from the haemolymph to the tubule in which an osmotic gradient for the passage of fluid is generated (Piermarini, Esquivel, and Denton 2017). $Na^+/K^+$ -ATPase (NKA) and V-type $H^+$ -ATPase (VA) are ionomotive pumps which are involved in driving epithelial transport processes in an organism (Patrick et al. 2006). VA is an electrogenic proton pump that generates energy in the apical and basolateral membranes in addition to the paracellular pathway, allowing for transepithelial secretion of KCl and NaCl in principal cells of the Malpighian tubules (Tiburcy, Beyenbach, and Wieczorek 2013; Emery and Billingsley 1998). VA pumps protons from the cytosol into the tubule lumen, creating a negative membrane voltage across the apical and basolateral membrane (Piermarini, Esquivel, and Denton 2017). The membrane voltage created by VA can drive ion transport through ion specific channels while its electrochemical proton potential can allow for secondary active transport through cation or anion/$H^+$ cotransport (Jonusaite, Kelly, and Donini 2011; Patrick et al. 2006). This voltage drives the uptake of $Na^+$ and $K^+$ from the haemolymph, while on the apical membrane it is suggested to facilitate the uptake of protons in exchange for $Na^+$ or $K^+$ excretion via cation-proton antiporters (Piermarini and Gillen 2015). NKA allows for the maintenance of transmembrane electrochemical potential differences which are critical in secondary transport and cell signaling (Emery and Billingsley 1998). In *A. aegypti* Malpighian tubules, NKA is found to play a smaller role in ion transport, specifically in stellate cells in which it potentially contributes to the uptake of peritubular $K^+$ and expulsion of intracellular $Na^+$ across the basolateral membrane (Piermarini and Gillen 2015).

The Malpighian tubules of *A. aegypti* are not innervated, and diuresis is regulated by hormones (Tiburcy, Beyenbach, and Wieczorek 2013). Mosquito natriuretic peptide (MNP), a known stimulator of Malpighian tubule secretion acts via the second messenger cyclic AMP, to

stimulate secretion of $Na^+$(Coast et al. 2005; Donini et al. 2006; Petzel et al. 1987) . It is suggested that cAMP, and its membrane permeable analogs, depolarize the basolateral membrane voltage of the principal cells and hyperpolarize the transepithelial voltage in the Malpighian tubules. This is accompanied by reductions in transepithelial resistance and fractional resistance of the basolateral membrane, therefore contributing to an increase in $Na^+$ conductance across the basolateral membrane (Coast et al. 2005).

As mentioned, the main iono and osmoregulatory tissues in *I. scapularis* are the salivary glands in which NKA, in contrast to *A. aegypti* Malpighian tubules, plays a critical role. The salivary glands are innervated and known to be stimulated by dopamine, which induces saliva secretion (Kim et al. 2016). Two known dopamine receptors (D1and InvD1L) are found in the salivary glands, which when stimulated by dopamine, allow for both water-solute influx and pumping/gating during on-host feeding periods (Kim, Šimo, and Park 2014). Ouabain, a NKA inhibitor, terminates salivary gland secretions at high doses suggesting that NKA plays an important role in the downstream signaling pathway following dopamine stimulation. At low doses, ouabain only slightly inhibits saliva secretion, in addition to causing the secretion of a hyperosmotic saliva (Kim et al. 2016). The type III acini are suggested to allow for ion and osmoregulation during on-host feeding periods accounting for the majority of ions and fluids excreted back to the host simultaneously with the uptake of the blood meal. This is done through the activation of downstream signaling pathways involving dopamine receptor activation and the help of NKA (Kim et al. 2016). The type I acini allow for osmoregulation during off-host periods through the production of a hygroscopic saliva that makes its way to the surface of the hypostome, allowing for the absorption of atmospheric moisture (Francischetti et al. 2009). Due to their critical roles in the transmission of several pathogens causing disease, characterization of

the tick salivary glands has focused largely on their role in facilitating pathogen transmission. Research on the ability of the salivary glands to allow for ion and osmoregulation during both on-host and off-host periods is fairly novel, and thus the aim is to further characterize the salivary glands and the distribution of ionomotive pumps within them through immunohistochemical localization and enzyme activity assays.

The Malpighian tubules of *I. scapularis* have not been thoroughly characterized due to the primary role the salivary glands play in iono and osmoregulatory physiology in the tick. The Malpighian tubules of *I. scapularis,* in addition to the rectal sac, anal canal, and intestine, compose the tick hindgut. It is in the hindgut where metabolic wastes are excreted as guanine crystals (Sonenshine and Roe 2014). The contraction of circular and longitudinal smooth muscle cells in the intestine allows for the passage of haematin from the midgut to the rectal sac, and nitrogenous waste from the Malpighian tubules to the rectal sac (Sonenshine and Roe 2014). It is suggested that the rectal sac is involved in osmoregulation through the concentration of primary urine arriving from the Malpighian tubules (Campbell et al. 2010). The tick Malpighian tubules are localized at the junction of the intestine and the rectal sac, and are involved in the excretion of guanine crystals. In addition, the Malpighian tubules are characterized as having a brush border layer of microvilli, in addition to cells with a basal labyrinth on the outside facing the haemolymph, characteristic of cells which allow for fluid transport (Sonenshine and Roe 2014). It is suggested that the tubules may play a role in water elimination and ion resorption as their cellular makeup of vacuoles and mitochondria at the lumen allows for the active and passive transports of fluids and ions. A previous study carried out by Kaufman and Phillips (1983) on *Dermacentor andersoni,* a tick of the Ixodid family, found that saliva excreted back to the host during blood meal engorgement contained more $Na^+$ than $K^+$, with excess ions being excreted via

the anus and salivary glands. $K^+$ was found in excess in faecal waste compared to in salivary gland secretion and haemolypmh, suggesting two potential hypotheses (Kaufman and Phillips 1973). If host blood passes directly into the rectal sac via the midgut, the midgut may be involved in only transporting $Na^+$, $Cl^-$, and water into the haemolymph as it is potentially impermeable to $K^+$ given that $K^+$ levels are low in the haemolymph. A second hypothesis is that because $K^+$ is often found in the Malpighian tubule secretions of insects, perhaps the Malpighian tubules of ticks are involved in secreting it (Kaufman and Phillips 1973). The rectal sac is also suggested to be involved in ion and osmoregulation as previous studies have found that certain aquaporins can be found within the rectal sac of ticks (Ball et al. 2009). Aquaporin IrAQP1was found to be expressed and associated with tissues containing high water flux, such as the salivary glands and was also found in the hindgut and midgut (Campbell et al. 2010). The luminal lining of epithelial cells within the rectal sac also were found to have multiple microvilli which form a brush border, analogous to cells involved in water passage (Sonenshine and Roe 2014). In other blood feeding arthropods, such as *A. aegypti,* the ileum, rectum, and the Malpighian tubules serve primary roles in the ion and osmoregulation of the organism, specifically during blood meal engorgement. Comparatively, unlike the mosquito, the transport mechanisms and regulation of fluid and ion transport by the Malpighian tubules and rectal sac in ticks have not been characterized. Due to the fact that ionomotive pumps drive fluid transport, determining the distribution and activity of primary active transporters such as the NKA and VA will help to determine the role that both the Malpighian tubules and rectal sac play in ion and osmoregulatory processes in the tick, *I. scapularis*. This is particularly important as only a portion of the excess ions and fluids are excreted back to the host during blood meal engorgement via the salivary glands (Kaufman and Phillips 1973).

The midgut of *I. scapularis* is mainly involved in the processing, digestion and storage of the blood meal as ticks can hold their blood meal for weeks after engorgement (Sonenshine, 2014).The midgut is made up of two parts: a stomach (central ventriculus) and a numerous diverticula which extend from the stomach (Sonenshine and Roe 2014). The two types of cells within the midgut, the digestive and undifferentiated cells, are involved in ingesting haemoglobin and transporting haematin to the rectal sac for excretion of nitrogenous waste (Lara et al. 2005). The midgut undergoes morphological changes to allow for the digestion of a blood meal, to which its final product is digested food, with waste moving to the Malpighian tubules and rectal sac via the intestine (Sonenshine and Roe 2014).

The hindgut, Malpighian tubules, and midgut are organs making up part of excretory system of the blacklegged tick *I. scapulari*s, and are fairly uncharacterized. Given that it faces the challenge of regulating ion and fluid levels following blood meal engorgement as well over periods of fasting between feeding bouts, the objective of this study was to further characterize the tissues involved in iono- and osmoregulatory processes. Ionomotive pumps are known to drive ion transport and subsequently fluid transport between the gut lumen and the haemolymph, allowing for the excretion of excess ions and fluids, and thus maintaining haemolymph homeostasis (Piermarini and Gillen, 2015.; Piermarini, Esquivel, and Denton 2017; Patrick et al. 2006). This study aimed to determine the distribution of NKA and VA in the salivary glands, midgut, rectal sac, intestine, and Malpighian tubules. Immunohistochemistry was used to localize and determine the organ specific localization of NKA and VA. Colocalization of NKA and VA was found in specific acini types of the salivary glands, in addition to colocalization found throughout the Malpighian tubules. The regulation of the Malpighian tubules by prospective second messengers was examined in order to understand their contribution in regulatory

processes via the application of the Ramsay assay technique, in which Malpighian tubules are isolated *in vitro* within a droplet of saline containing test compounds. The proximal end of the tubule, which empties into the rectal sac, is placed outside the saline droplet under hydrated mineral oil and the rate of secretion can be examined (Ramsay, 1954). The potential role of cAMP and cGMP as second messengers leading to the stimulation of tick Malpighian tubule secretion was also determined, given that these factors act as a second messengers in the regulation of insect Malpighian tubules, and also for cAMP-mediated dopamine stimulation of *I. scapularis* salivary glands. NKA and VA activities in the midgut, hindgut (rectal sac, Malpighian tubules, intestine), and salivary glands were also examined in order to quantify the activity of these ionomotive pumps in different gut regions.

## Materials and Methods

### Experimental Animals

Adult female and male *Ixodes scapularis* used for all experiments were obtained from the Tick Rearing Facility at Oklahoma State University, Oklahoma, United States.

### Dissections

Dissections of *I. scapularis* were carried out in a saline earlier described for *Rhodnius prolixus*; 129mM NaCl, 8.6 mM KCl, 8.5 mM $MgCl_2$, 2.0 mM $CaCl_2$, 10.2 mM $NaHCO_3$, 4.3 mM $NaH_2PO_4$, 8.6 mM HEPES, 20 mM glucose; pH 7.0 with 500 mL dd $H_2O$ (O'Donnell 2012; Gardiner 1983). *R. prolixus* saline was used due both *I. scapularis* and *R. prolixus* being hematophagous arthropods. Dissections were carried out on dental wax in which *I. scapularis* was placed ventral side down. The dorsal cuticle was removed along with inner trachea and connective tissue to expose the salivary glands, Malpighian tubules, midgut, intestine, and rectal sac. Each desired organ was then removed using fine forceps and glass probes.

### Immunohistochemical Localization of NKA

To determine the presence of NKA in various organs, both female and male adult *I. scapularis* (between 1-3 months after moulting) were collected and immunoreactivity was tested. The salivary glands, Malpighian tubules, midgut, intestine, and rectal sac were dissected under saline and removed using fine forceps. NKA was localized in tissues of *I. scapularis* using a mouse monoclonal antibody raised against the alpha-subunit of avian NKA ($\alpha$5; Developmental Studies Hybridoma Bank, Iowa City, IA, USA) at a dilution of 1:10. Tissues were fixed in 4% paraformaldeyhyde solution at 4°C overnight and were then rinsed with 1x phosphate buffered saline (PBS) before being digested with 4% Triton X-100 for one hour. Tissues were then washed with 1x PBS and incubated with the mouse NKA antibody for 48 hours at 4 °C. Tissues

were then washed and placed in 1x PBS at 4 °C overnight. Tissues were then incubated with a goat anti-mouse Alexa Fluor 647 (1:200 dilution) (Life Technologies, Burlington, ON), and 0.165 μM phalloidin conjugated to Alexa Fluor 488 (Life Technologies, Burlington, ON) overnight at 4 °C. Tissues were washed with 1x PBS the following day, and then mounted on slides with 50% glycerol containing DAPI. Controls omitting the primary antibody were conducted to confirm the absence of non-specific binding and was completed for all immunohistochemical analyses. Slides were analyzed using confocal microscopy, using a ZEISS LSM 700 microscope (Carl Zeiss, Inc, United States)

**Immunohistochemical Localization of VA**

The same procedure was followed as previously described, however tissues were stained using a mouse anti-ATP (Cat# H00000535-A01, Abnova Corporation, Taiwan) at a dilution of 1:500. Tissues were then incubated with a goat anti-mouse Alexa Fluor 647 (1:200 dilution) (Life Technologies, Burlington, ON), and 0.165 μM phalloidin conjugated to Alexa Fluor 488 (Life Technologies, Burlington, ON) overnight at 4 °C, washed 3 times for 15 minutes at room temperature, and mounted on slides with mounting media containing 50% glycerol and DAPI. Controls omitting the primary antibody were conducted. Slides were analyzed using confocal microscopy, using a ZEISS LSM 700 microscope (Carl Zeiss, Inc, United States).

**Immunohistochemical Localization of VA and NKA**

In order to examine the distribution of both VA and NKA, immunohistochemistry was completed first for NKA, as described in the procedure above. The secondary antibody used was an anti-mouse Alexa Fluor 647 at a 1:200 dilution (Life Technologies, Burlington, ON). To enable co-detection of VA, a guinea pig antibody generated against the v1 complex of VA of *Manduca sexta* was utilized, a kind gift from Dr. Wiczorek, Universität Osnabrück, Germany

(antibody 353-2) (Weng et al. 2003) . The secondary antibody was a goat anti-guinea pig Alexa Fluor 488 (Life Technologies, Burlington, ON) which was used at a dilution of 1:500. Tissues were washed 3 times for 15 minutes at room temperature, mounted on slides containing mounting medium of 50% glycerol and DAPI. Slides were analyzed using confocal microscopy, using a ZEISS LSM 700 microscope (Carl Zeiss, Inc, United States).

**Dopamine stimulated immunohistochemical localization of VA and NKA**

Dopamine activates salivary gland secretion via two dopamine receptors expressed in the salivary glands. To determine if dopamine stimulation influences VA and NKA localization, immunoreactivity of VA and NKA in the salivary glands was examined after dopamine treatment. Salivary glands were placed into a droplet of $10^{-6}$ M dopamine and left to incubate for 15 minutes, similar to treatments described previously (Sauer et al. 1995; Just et al. 1996; Donghun Kim, Šimo, and Park 2014). Following incubation, salivary glands were isolated and fixed in 4% paraformaldehyde solution at 4°C overnight. Immunohistochemistry was completed as described above for VA and NKA antibodies. Controls omitting the primary antibody were conducted to confirm the absence of non-specific binding. Control unstimulated and dopamine stimulated salivary glands were analyzed using confocal microscopy, using a ZEISS LSM 700 microscope (Carl Zeiss, Inc, United States).

**Malpighian tubule secretion assays**

Ramsay assays were performed as previously described for insects (Ramsay 1954) on isolated *I. scapularis* Malpighian tubules, which are a single pair attached at the intestine-hindgut junction (Sonenshine and Roe 2014). All experiments were performed on Malpighian tubules isolated from unfed adult male and female *I. scapularis* (between 2-4 months after moulting). Dissections were completed with the ticks secured onto dental wax. Tubules were removed and

placed into a 20 µL droplet of *Rhodnius prolixus* saline on a Sylgard coated Petri dish. The Petri dish was filled with hydrated mineral oil to submerge the 20 µl droplets of saline containing the Malpighian tubules. The proximal ends of the Malpighian tubules empty out into the junction of the hindgut and rectal sac, while the distal ends are free floating in the haemolymph. The distal end of the tubules was placed into the saline droplet, while the proximal end was placed on a minutin pin outside of the saline droplet within the hydrated mineral oil.

**Calculating secretion rate**

Secretion rate of Ramsay assays (Ramsay 1954) were calculated via obtaining the diameter of the secreted droplet at the proximal end of the Malpighian tubule. To calculate volume of the secreted droplet, the following equation was used:

$$V = (\pi/6)d^3$$

where d is diameter, assuming the droplet is spherical, and V is volume of the secreted droplet. To calculate the secretion rate in nL/min, the volume was divided by the time in minutes

$$V = (\pi/6)d^3 \,/\, \text{time (min)}$$

In all experiments, measurements of secreted droplet diameter were obtained after 30 minutes.

**Secretion assays treatments**

Malpighian tubules were dissected out and placed into a saline droplet as described above. The proximal end was attached to a pin outside of the droplet, submerged in hydrated mineral oil. Malpighian tubules were stimulated with either 8-bromoadenosine 3’, 5’-cyclic monophosphate or 8-bromoguanosine 3’, 5’-cyclic monophosphate (Sigma-Aldrich, Oakville, ON, CA), which are membrane permeable analogs of cAMP and cGMP, respectively. 2 µL of saline was removed from the saline droplet. A 1M membrane permeable 8-bromoadenosine 3’,5’-cyclic monophosphate (8-bromo cAMP) stock was diluted ten-fold by adding 2 µL into the

18 uL saline droplet containing the Malpighian tubule, to achieve a final concentration of 0.1 M 8-bromo cAMP. The same procedure was done for membrane permeable 8-bromoguanosine 3’,5’-cyclic monophosphate (8-bromo cGMP). Malpighian tubules were left in each treatment for 30 minutes before recording the diameter of the secreted droplet. Control treatments were done on the same day as experimental treatments using separate tubules of different ticks by leaving the Malpighian tubules submerged in saline for 30 minutes before measuring the diameter of the secreted droplet.

**NKA and VA Activity Assays**

The salivary glands, midgut, and hindgut (intestine, rectal sac, and Malpighian tubules) were dissected from adult male and female *I. scapularis* (1 month after moulting), and were flash frozen in liquid nitrogen and stored in -80°C. VA and NKA were determined using a modified 96-well microplate method described in Jonusaite et al. (2011), which was based on the technique of McCormick (1993). Specifically, this assay relies on enzymatic coupling of VA and NKA inhibitors, namely bafilomycin (LC Laboratories, Woburn, MA, USA) and ouabain (Sigma- Aldrich Canada, Ltd.), and hydrolysis of adenosine triphosphate to the oxidation of reduced nicotinamide adenine dinucleotide (NADH), whose decrease can be measured in a spectrophotometer. Ten samples of each tissue were collected into microcentrifuge tubes and samples were kept on ice and combined with homogenization SEID buffer composed of four parts SEI (150mM sucrose, 10mM EDTA, and 50 mM Imidazole; pH 7.3) and one part 0.5% sodium deoxycholic acid (Jonusaite, Kelly, and Donini 2011). Samples were then homogenized on ice using a PRO 250 homogenizer with a 5x75 mm generator (PRO Scientific Inc.) for 10 seconds. Homogenized tissues were then centrifuged in a benchtop centrifuge at 10,000*g* for 10 minutes at 4°C. The supernatants from each sample were then further separated into 20 µL

aliquots in 1.5 mL centrifuge tubes, and stored at -80°C. Solutions for the enzyme assay were prepared with the following compositions: *Solution A* (4 units/mL lactate dehydrogenase (LDH), 5 units/mL pyruvate kinase (PK), 2.8mM Phosphoenolpyruvate (PEP), 3.5mM ATP, 0.22mM NADH and 50mM imidazole) prepared in de-ionized water and titrated to pH 7.5, stored at 4°C; *Solution B* (*Solution A* with 5μM ouabain (Sigma-Aldrich, Oakville, ON, CA)); *Solution C* (*Solution A* with 10 μM bafilomycin). To make a working solution from stock solution A, solution A was combined with a salt solution (189 mM NaCl, 10.5mM $MgCl_2$) in a 3:1 ratio of stock solution to salt before preparing Solution B and C. All solutions were kept on ice throughout the experiment. Ouabain and bafilomycin concentrations were previously determined based on a comparison of the ATPase activities resulting from the inhibition of NKA activity with a variety of ouabain and bafilomycin concentrations (Jonusaite, Kelly, and Donini 2011).

A standard curve using adenosine diphosphate (ADP) (Sigma-Aldrich, Oakville, ON, CA) was completed before the assay was run with tissue sample extracts to ensure that assay solutions A, B and C were suitable. ADP standards were made using 4mM ADP and a 50mM imidazole buffer (IB) (pH 7.5). Five standards of ADP were prepared: 0 nM (200 μL IB), 5nM (25 μL 4mM ADP, 175 μL IB), 10 nM (50 μL 4mM ADP, 150 μL IB), 20 nM (100 μl 4mM ADP, 100 μL IB), and 40 nM (200 μL 4mM ADP). Aliquots (10 μL) of each standard was loaded in duplicates onto a 96-well microplate in addition to 200 μL of *Solution A*. The plate was then placed in a Synergy2 Multi-Mode Microplate Reader (BioTek, Winooski, VT) at room temperature and a linear rate of NADH disappearance was obtained by measuring absorbance at 340 nm. The expected optical density readings (OD) for 0-40 nm ADP standards were between 0.9 and 0.2 OD. The slope should be between -0.012 to -0.014 OD nmol ADP. If the slope was between expected values, the samples were thawed on ice and loaded onto the microplate kept

cool on ice in five replicates. Samples of each tissue (collected in five biological replicates) were loaded in duplicates and filled with 200 µL of working *Solution A*, working *Solution B*, and working *Solution C*. NADH absorbance decrease was measured for 30 minutes at 340 nm.

**NKA and VA activity**

The activity of NKA and VA from the enzyme assay were calculated with the following equation:

**NKA or VA activity = ((change in ATPase/S) /[P]) x 60 (min)**

Where change in ATPase is the difference in ATP hydrolysis in the absence and presence of ouabain or bafilomycin. S is the slope obtained from the ADP standard curve. [P] is the protein concentration of the sample (obtained via Bradford Assay). The final activity was expressed as µmol ADP per mg of protein per hour.

**Bradford Assay**

The Bradford assay was completed in order to determine protein concentration of samples for the enzymatic assay measuring NKA and VA activities. The protein content of each sample was determined using the Bradford assay reagent (Sigma-Aldrich, Oakville, ON, CA) according to the manufacturers guidelines with bovine serum albumin (Bioshop Canada Inc., Burlington, ON, CA) as a standard.

**Statistical Analysis**

Data were plotted using Graphpad Prism 6 (Graphpad Software Inc., La Jolla, CA), and all values were expressed as mean ± SEM. Ramsay assay secretion rates were analyzed using an unpaired Student's t-test. NKA and VA activities were analyzed using a paired Student's t-test. Data were significant if $p<0.05$.

**Results**

To determine the organ-specific distribution of ionomotive pumps NKA and VA, immunohistochemistry was carried out in the salivary glands, midgut, intestine, rectal sac, and Malpighian tubules of *I. scapularis*.

Immunohistochemical localization of NKA, VA, and f-actin in the salivary glands

NKA immunoreactivity was mainly found in the type I acini and was also observed in the type III acini, however at a lower intensity (Figure 4a, 5, 6). VA was observed mainly in type II and type III acini (Figure 4, 5, 6). VA was colocalized with NKA in the type III acini (Figure 6). When stimulated by dopamine, male and female salivary glands were found to have different immunohistochemical distribution of the ATPases (Figure 4, 5). Dopamine stimulation of male salivary glands appears to cause a greater distribution of VA throughout the different acini types (Figure 5b), when compared to unstimulated salivary glands (Figure 5a). In contrast, the dopamine stimulation of female salivary glands causes an increase in NKA distribution (Figure 4b), in comparison to unstimulated salivary glands (Figure 4a). Phalloidin immunoreactivity was observed towards the border of the type III acini where the ablumenal insterstitial cell lies, while it was found at the base of type II acini close to the alveolar duct (Figure 6).

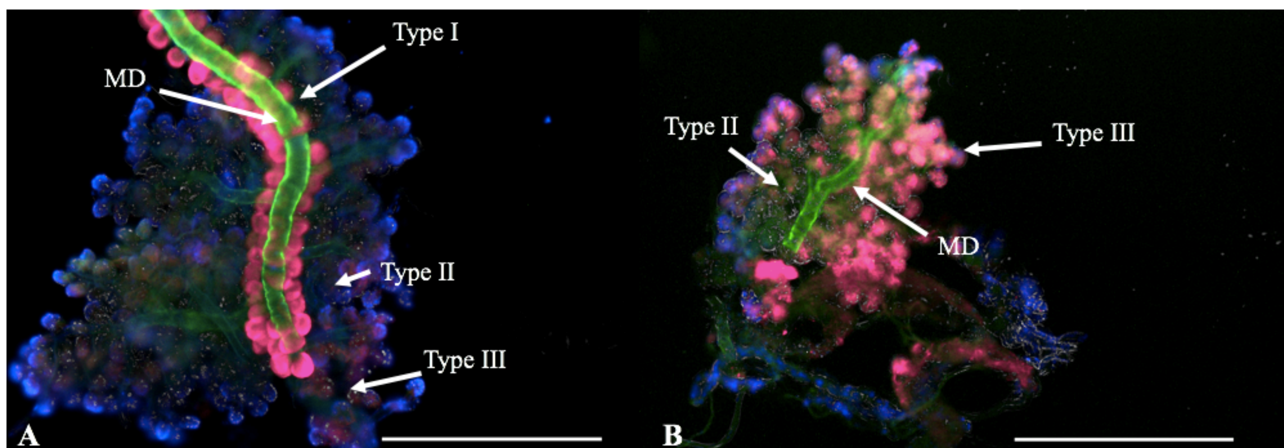


**Figure 4. Immunoreactivity of unstimulated and dopamine-stimulated salivary glands of adult female *I. scapularis***. **(A)** Unstimulated female salivary glands show $Na^+/K^+$ ATPase immunoreactivity (pink) in type I acini while type III acini also show some immunoreactivity. Type II and the main duct (MD) show V-type $H^+$ ATPase immunoreactivity (green). **(B)** Dopamine-stimulated female salivary glands show an increase in $Na^+/K^+$ ATPase specifically in type III acini (pink). Images are overlays Alexa Fluor 647 (NKA), Alexa Fluor 488 (VA), and DAPI (Nuclei). Scales are 200 μm **(A)** and 400 μm **(B)**.

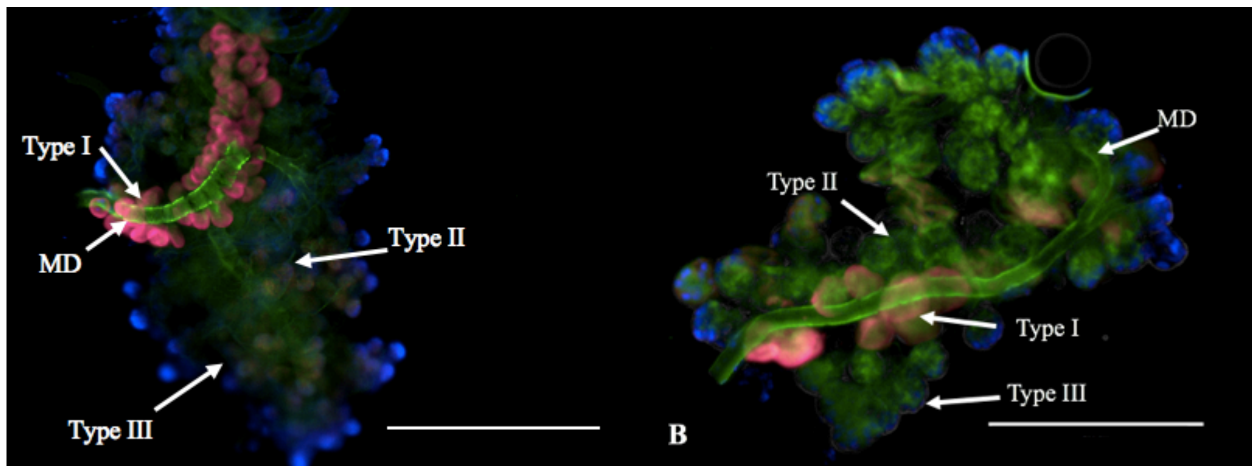


**Figure 5. Immunoreactivity of unstimulated and dopamine-stimulated salivary glands from adult male *I. scapularis*.** $Na^+/K^+$ ATPase (pink) and V-type $H^+$ ATPase (green), and DAPI (blue) in the salivary glands**. (A)** $Na^+/K^+$ ATPase in unstimulated salivary glands is observed mainly in type I acini while V-type $H^+$ ATPase is observed in type II and III acini. **(B)** $Na^+/K^+$ ATPase immunoreactivity in dopamine-stimulated salivary glands from males appears the same as unstimulated. In contrast, V-type $H^+$ ATPase is more intense throughout all acini type, including type I. Images are overlays Alexa Fluor 647 (NKA), Alexa Fluor 488 (VA), and DAPI (Nuclei). Scales are 200 μm.

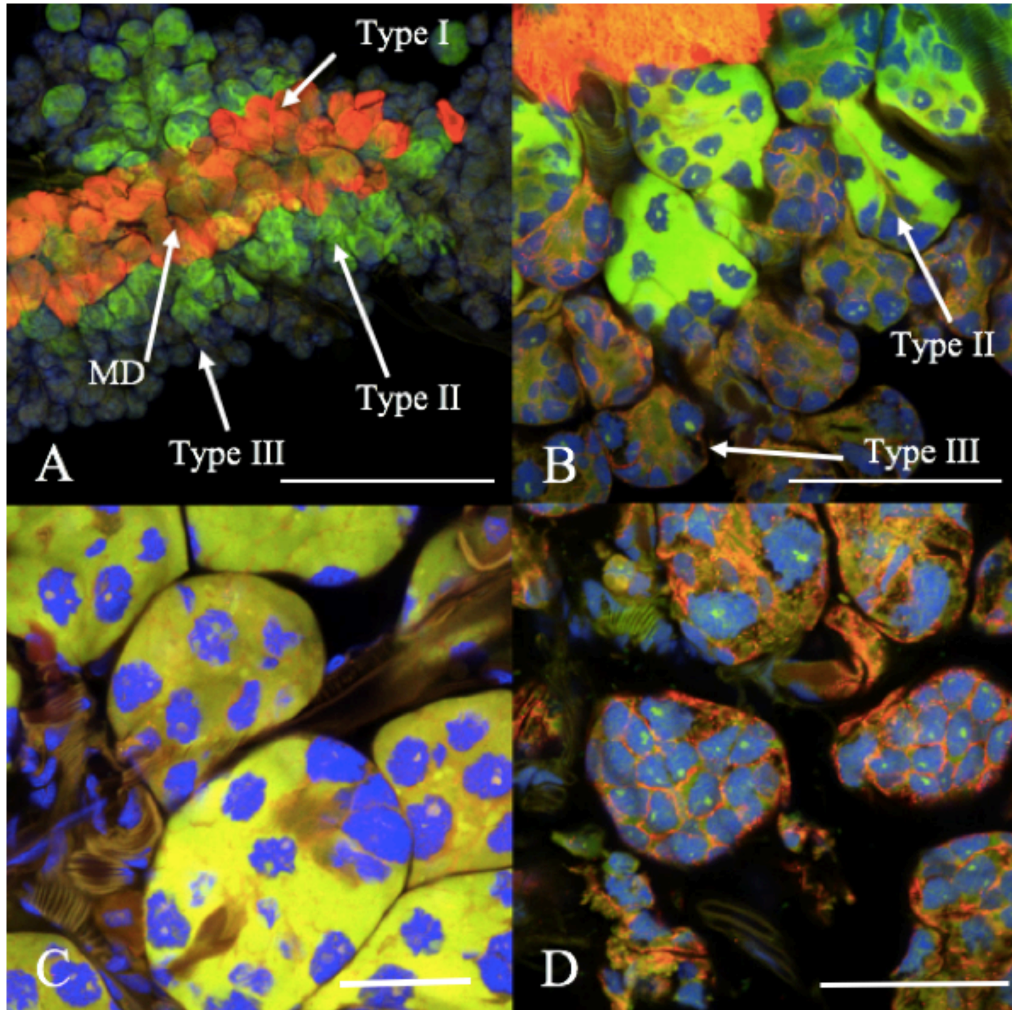


**Figure 6. Immunoreactivity of $Na^+/K^+$ ATPase (red) and V-type $H^+$ ATPase (green), and DAPI (blue) in the salivary glands *I. scapularis* adults. (A).** Positive immunoreactivity of $Na^+/K^+$ ATPase is seen in epithelial cells of all three acini types although is mainly observed in type I acini around the main salivary duct (MD). **(B, C)** Co-localization (yellow) of V-type $H^+$ ATPase (green) and $Na^+/K^+$ ATPase (red) is seen in type II acini at higher magnifications. **(D)** $Na^+/K^+$ ATPase immunoreactivity is seen around cells (in membrane) within type III acini along with V-type $H^+$ ATPase co-localization. The MD shows positive staining for V-type $H^+$ ATPase. Blue exemplifies nuclear staining. Images are overlays of Alexa Fluor 647 (NKA), Alexa Fluor 488 (VA) and DAPI (Nuclei) **(A-D)**. Scales provided are 400 µm **(A,B)**, 100 µm (**C)** and 200 µm **(D)**.

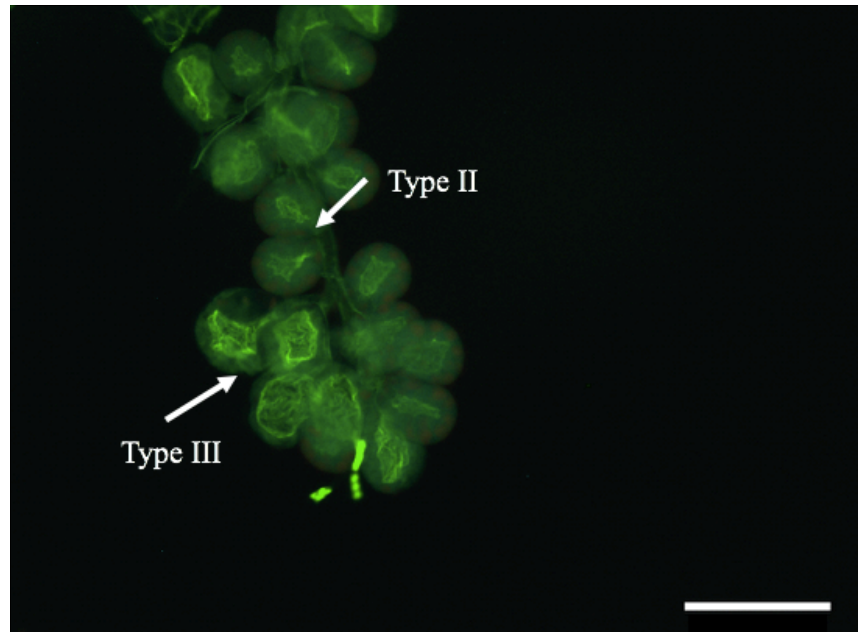


**Figure 7. Immunoreactivity of f-actin in type II and III acini in adult *I. scapularis* salivary glands.** Type III acini shows f-actin staining towards the apical surface of the acini where the abluminal insterstitial cell lies, while type II acini show f-actin immunoreactivity at the base of the type II acini where the the alveolar duct is. Image is an overlay of Alexa Fluor 488 (phalloidin conjugated to f-actin). Scale is 100 µm.

Immunohistochemical localization of NKA, VA, and f-actin in the midgut

The distribution of NKA and VA in *I. scapularis* midgut was determined using immunohistochemistry by examining immunoreactivity of NKA and VA. F-actin immunoreactivity was also examined to further characterize the organ, as a potential marker for musculature. The midgut is composed of the main portion (stomach) and midgut diverticula protruding out of the stomach. NKA immunoreactivity was observed to be evenly distributed along the midgut diverticula length (Figure 8a). Notably, however, less immunoreactivity was seen in the stomach, suggesting differential roles of NKA in these distinct gut regions (Figure 8b). VA is found in the stomach evenly distributed (Figure 9a); however, immunoreactivity decreases toward the distal portion of the midgut diverticula (Figure 9b). F-actin immunoreactivity was observed in a circular pattern along the length of the midgut diverticula, indicative of circular musculature only in this gut region (Figure 8). F-actin immunoreactivity was observed in the midgut stomach, in an unorganized circular and longitudinal pattern suggestive of circular and longitudinal musculature (Figure 8).

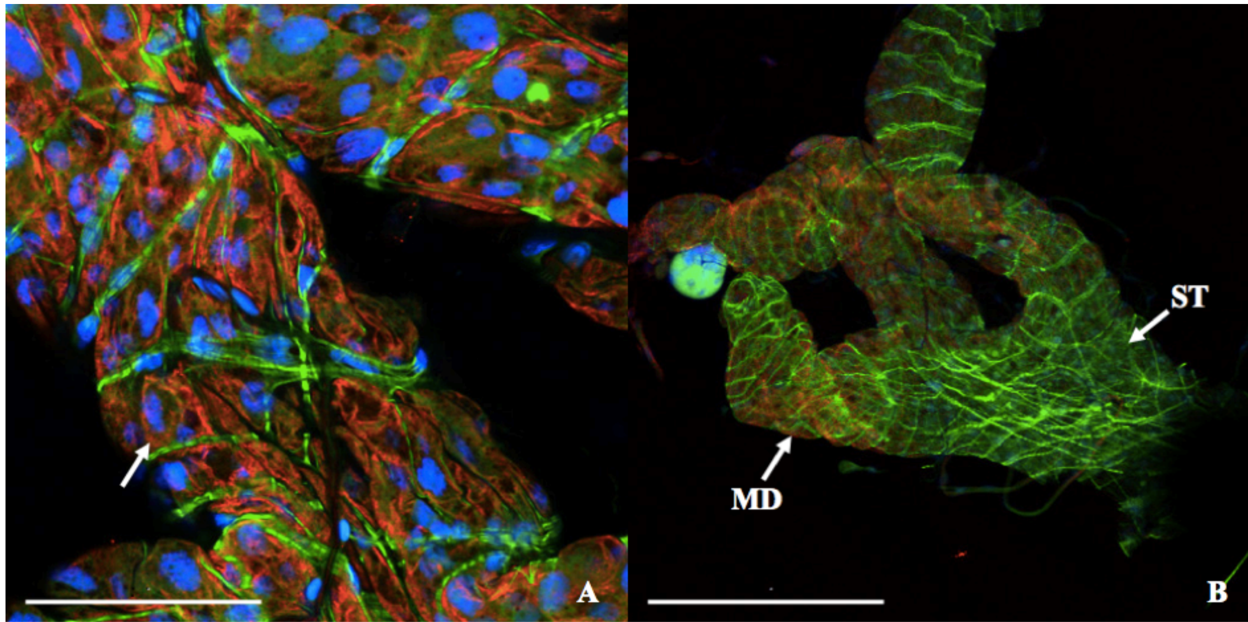


**Figure 8. $Na^+/K^+$ ATPase and f-actin immunoreactivity in the midgut and midgut diverticula of adult *Ixodes scapularis*. (A)** $Na^+/K^+$ ATPase (red) immunoreactivity in the midgut diverticula (MD), with immunoreactivity around the border of cells (denoted with arrows). F-actin immunoreactivity (green) is also seen in a circular pattern along the MD. **(B)** $Na^+/K^+$ ATPase throughout the midgut stomach (ST) and MD. F-actin immunoreactivity is seen along the diverticula of the midgut. Images are overlays Alexa Fluor 647 (NKA), Alexa Fluor 488 (phalloidin conjugated to f-actin), and DAPI (Nuclei). Scales are and 200 µm **(A)** and 400 µm **(B)**.

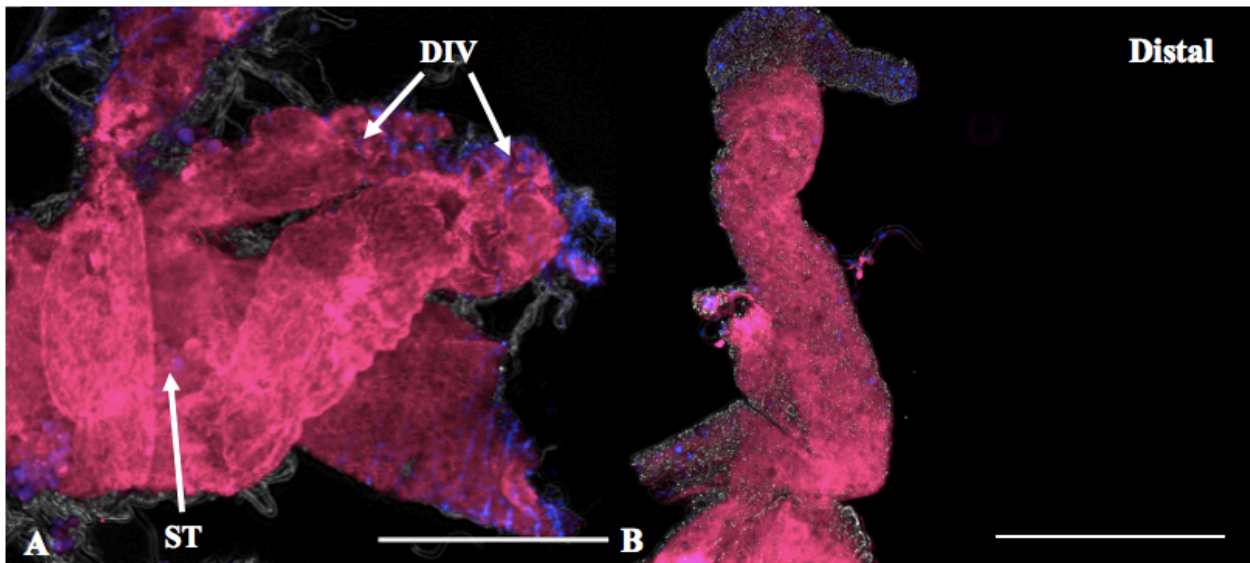


**Figure 9. V-type $H^+$ ATPase immunoreactivity in the midgut stomach and midgut diverticula of adult *Ixodes scapularis*. (A)** V-type $H^+$ ATPase (pink) immunoreactivity in the midgut stomach (ST) and diverticula (DIV). **(B)** V-type $H^+$ ATPase throughout the midgut diverticula. VA immunoreactivity decreases toward the distal end of the diverticula. Images were overlays of brightfield, Alexa Fluor 647 (NKA) and DAPI (Nuclei). Scales are 400 µm.

Immunohistochemical localization of NKA, VA, and f-actin in the hindgut

The distribution of NKA and VA in the *I. scapularis* hindgut (rectal sac, intestine, and Malpighian tubules) was determined using whole mount immunohistochemistry by examining immunoreactivity of NKA and VA. F-actin immunoreactivity was used as a potential marker for musculature to further characterize the hindgut composition.

*Intestine and rectal sac*

Staining for NKA was observed more so in the intestine than in the rectal sac (Figure 10a, 11). From the experiments, it appears guanine crystals elicit strong autofluorescence and thus the strong fluorescence observed within the rectal sac is not a true representation of NKA immunoreactivity. When immunohistochemical images of the rectal sac were devoid of guanine crystals, NKA immunoreactivity appeared less abundantly. Thus NKA immunoreactivity is less intense in the rectal sac compared to the intestine. F-actin immunoreactivity is strongly apparent in a circular and longitudinal pattern in the intestine, while in the rectal sac, immunoreactive staining was far less intense, with a poorly defined composition appearing mainly in a circular pattern (Figure 10b). VA immunoreactivity is not as intense in the intestine (Figure 11b). In contrast, VA immunoreactivity is observed more in the rectal sac (Figure 11b), suggesting differential roles of NKA and VA in the intestine and rectal sac regions of the hindgut.

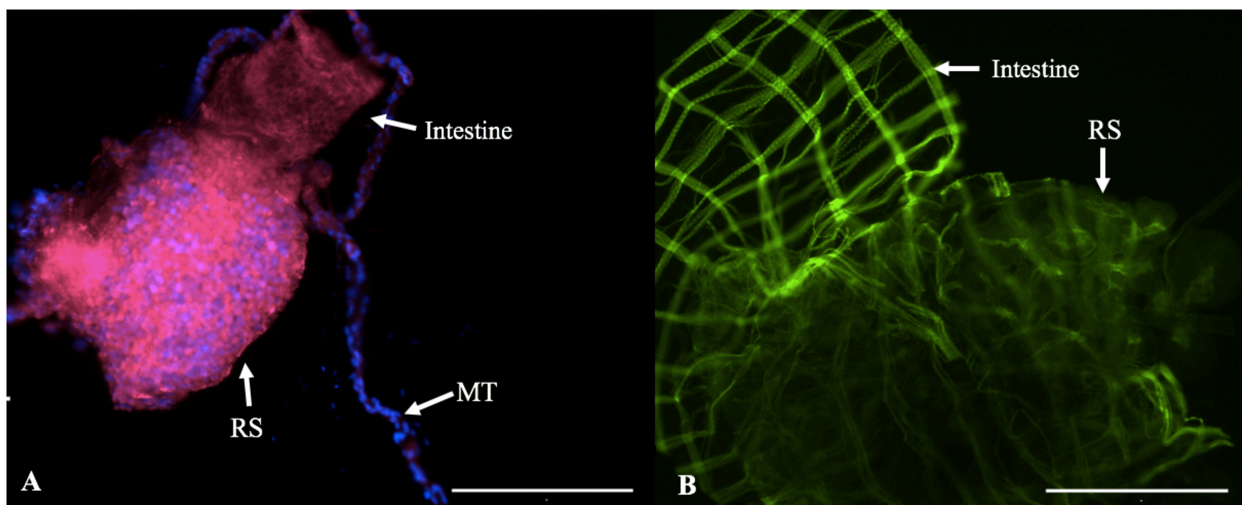


**Figure 10. $Na^+/K^+$ ATPase and f-actin immunoreactivity in the intestine and rectal sac of adult *Ixodes scapularis*. (A)** $Na^+/K^+$ ATPase (pink) immunoreactivity in the intestine has more intense staining as this rectal sac preparation is filled with guanine crystals which exhibit autofluorescence. **(B)** F-actin immunoreactivity (green) is seen in grid like pattern in the intestine and less intensely and poorly organized in the rectal sac (RS). MT denotes Malpighian tubule. Images are overlays Alexa Fluor 647 (NKA) and DAPI (Nuclei) **(A)**, Alexa Fluor 488 (VA) and DAPI **(B)**. Scales are 400 µm **(A)** and 200 µm **(B)**.

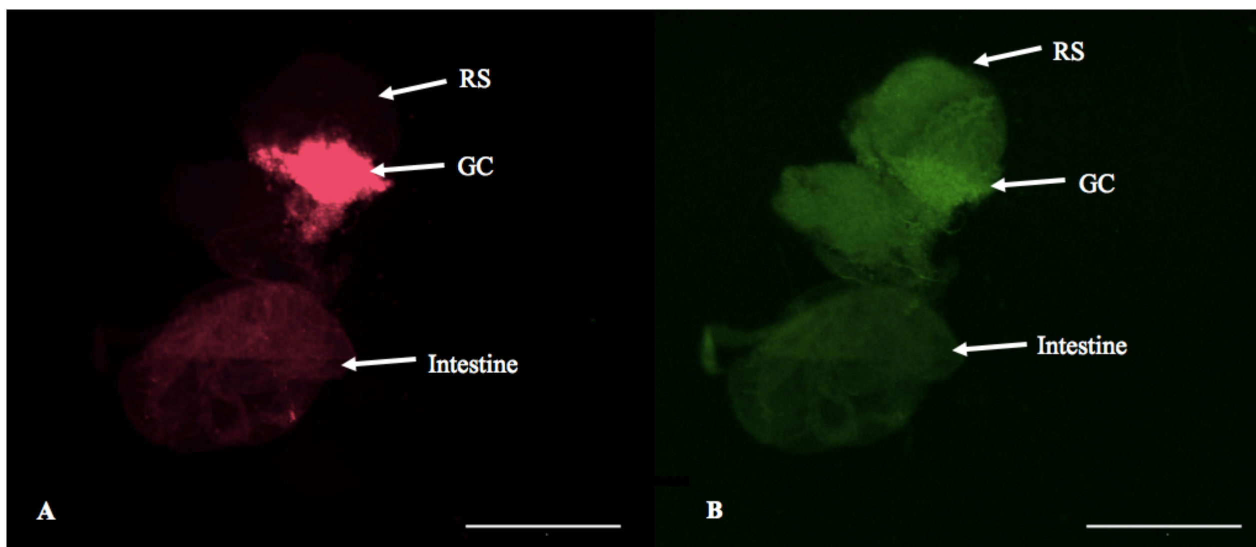


**Figure 11. $Na^+/K^+$ ATPase and V-type $H^+$ ATPase immunoreactivity in the intestine and rectal sac of adult *Ixodes scapularis*. (A)** $Na^+/K^+$ ATPase (pink) immunoreactivity in the intestine and rectal sac (RS). The intestine has more intense staining relative to the rectal sac, which is filled with guanine crystals (GC). **(B)** V-type $H^+$ ATPase (green) is observed in the rectal sac and intestine. Images are overlays of Alexa Fluor 647 (NKA) **(A)** and Alexa Fluor 488 (VA) **(B)** Scales are 400 µm **(A-B)**.

Immunohistochemical localization of NKA, VA, and f-actin in the Malpighian tubules

NKA immunoreactivity is observed along the Malpighian tubule length (Figure 12) and VA staining is observed throughout the Malpighian tubules (Figure 13).VA immunolocalization increases towards the proximal end of the Malpighian tubule (Figure 13). F-actin immunoreactivity is observed throughout the Malpighian tubule in a circular pattern, in addition to around the border of individual cells (Figure 12b). F-actin immunoreactivity is also observed in a grid like pattern in the intestine, at the most proximal portion of the Malpighian tubule length, where the tubules connect to the gut at the intestine-rectal sac junction (Figure 13b). Notably, when tubules were isolated into a saline droplet to complete the Ramsay assay (detailed in the subsequent section), the tubules were observed to be contracting. This coincides with the finding of f-actin immunoreactivity in a circular pattern throughout the tubule length (Fig 12B). Contractions were consistent and most prominently noticed in the proximal end of the tubule, close to where it forms a junction with the intestine and rectal sac.

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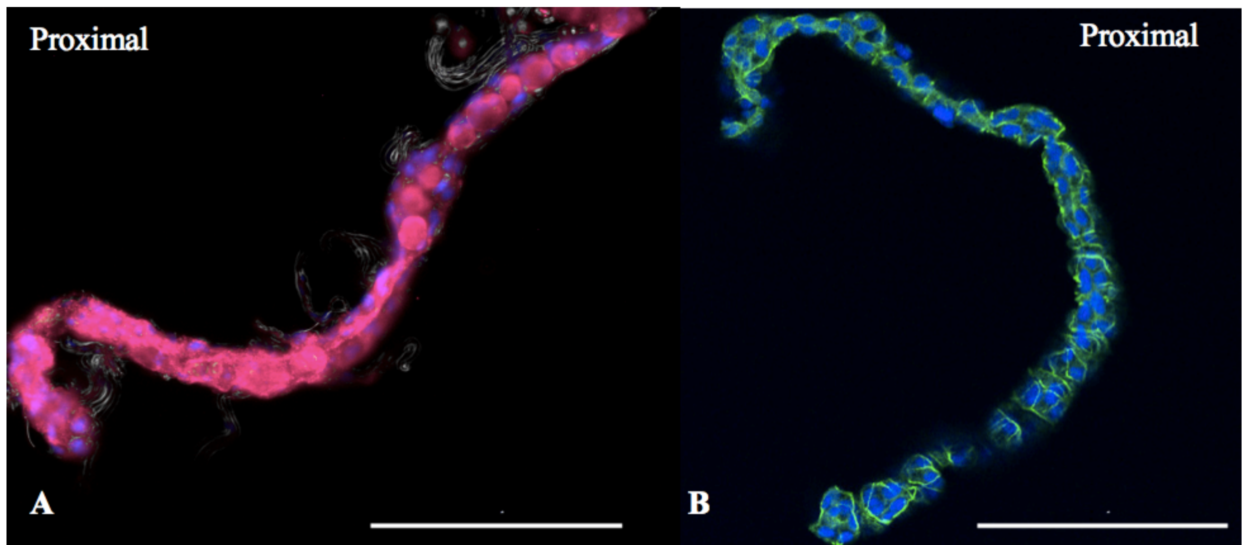


**Figure 12. $Na^+/K^+$ ATPase and f-actin immunoreactivity in the Malpighian tubules of adult *Ixodes scapularis*. (A)** $Na^+/K^+$ ATPase (pink) immunoreactivity in the Malpighian tubule. **(B)** F-actin immunoreactivity (green) is seen in a circular pattern along the tubule length. Images were overlays Alexa Fluor 647 and DAPI **(A)**, Alexa Fluor 488 and DAPI **(B)**. Scales are 400 µm.

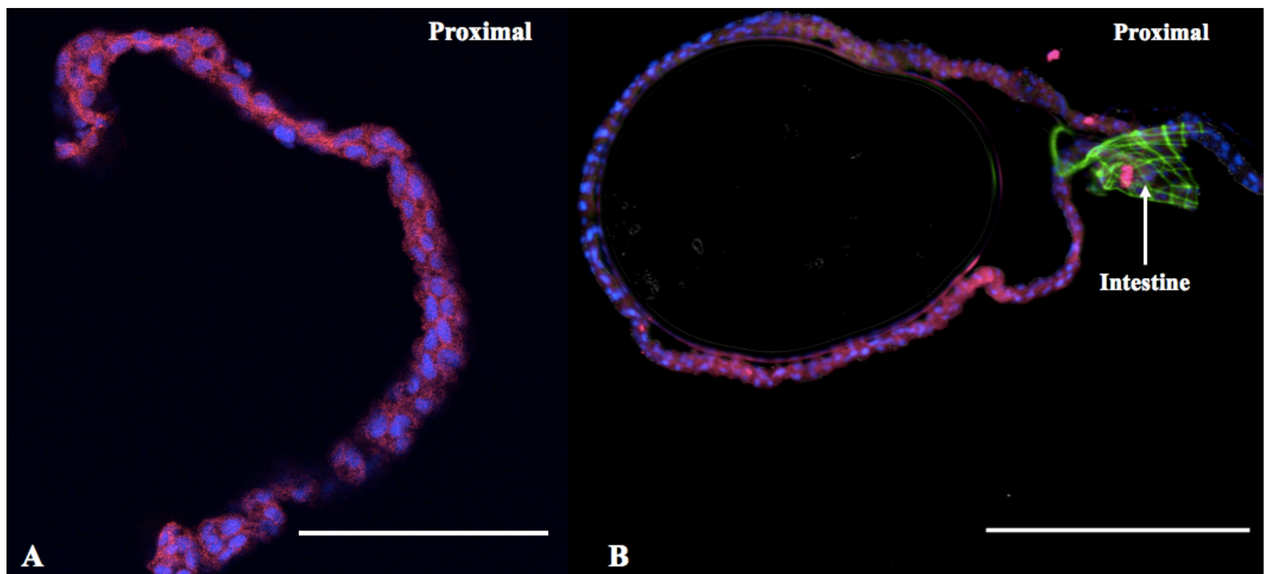


**Figure 13. V-type $H^+$ ATPase and f-actin_immunoreactivity in the Malpighian tubules of adult *Ixodes scapularis*. (A)** V-type $H^+$ ATPase (pink) immunoreactivity in the Malpighian tubule. **(B)** V-type $H^+$ ATPase (pink) immunoreactivity throughout the tubule length. F-actin immunoreactivity (green) is seen in a circular and longitudinal pattern at the most proximal portion which denotes the intestine. Proximal end of the tubule is labeled. Images are overlays Alexa Fluor 647 (VA), Alexa Fluor 488 (phalloidin conjugated to f-actin), and DAPI (Nuclei). Scales are 200 µm **(A)** 400 µm **(B)**.

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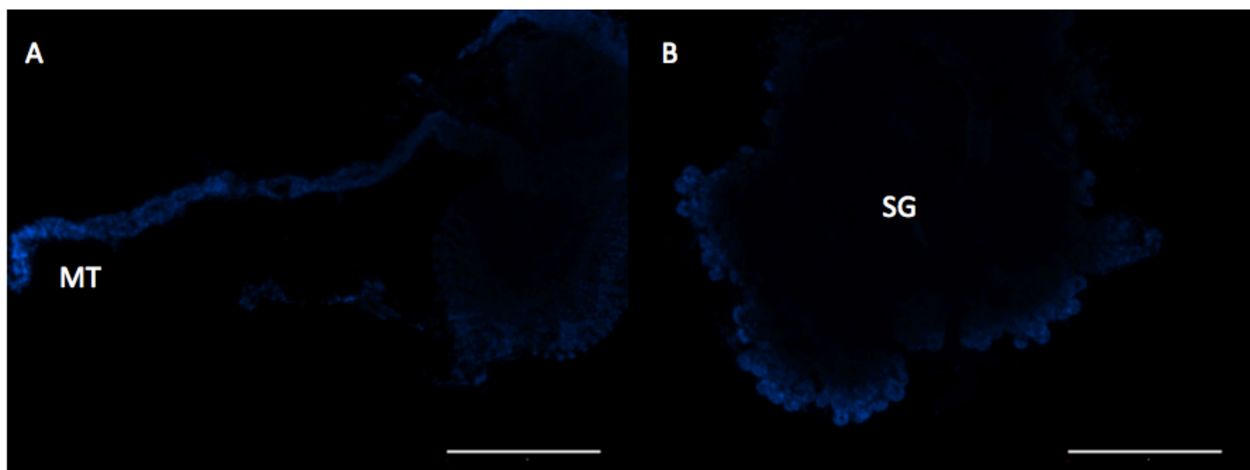


**Figure 14. Control treatments for V-type $H^+$ ATPase and $Na^+/K^+$ ATPase in the salivary glands and Malpighian tubules of adult *Ixodes scapularis*.** Immunohistochemistry was done omitting primary antibodies and using only secondary antibodies Alexa Fluor 488 on Malpighian tubules (MT) **(A)** and Alexa Fluor 647 on salivary glands (SG) **(B)**. No immunoreactivity is seen suggesting the immunofluorescence observed in the experimental trials is indicative of $Na^+/K^+$ ATPase and V-type $H^+$ ATPase distribution within the organs. Images are overlays Alexa Fluor 488 (VA) **(A)**, Alexa Fluor 647 **(A)**, and DAPI (blue) (Nuclei). Scales are 400 µm.

Malpighian tubule secretion assays

The Ramsay assay method allows for the *in vitro* stimulation of Malpighian tubules and the subsequent monitoring of fluid secretion rates, as well as the analysis of urine composition (Ramsay 1954). This study aimed to take some of the first steps in determining if *I. scapularis* Malpighian tubules are sensitive to secretagogues known to influence insect Malpighian tubules. This would be useful in order to better understand the control of the tick Malpighian tubules, including the activity of the second messengers cAMP and cGMP, to discover their contribution in ion and osmoregulation. Malpighian tubules were placed into a droplet of saline resembling the haemolymph and were left for 30 minutes in order to determine unstimulated secretion rates. The average unstimulated secretion rate was determined to be 0.0142 ± 0.0054 nL/min (Figure 15). Application of 0.1M 8-bromo cAMP stimulated the Malpighian tubules to secrete at a rate of 0.0579 ± 0.0273 nL/min (Figure 15), which was significantly greater than control secretion rates. Therefore, 0.1M 8-Bromo cAMP is seen to have stimulatory effects over four-fold greater than the unstimulated secretion rates which were determined in separate isolated Malpighian tubules.

The application of 0.1M 8-bromo cGMP was also found to promote Malpighian tubule secretion. Specifically, when the Malpighian tubules were placed in a control saline in this set of experiments, the unstimulated secretion rate was 0.0153 ± 0.0027 nL/min (Figure 16). Application of 0.1M 8-bromo cGMP produced significant stimulatory effects on the Malpighian tubules resulting in a secretion rate of 0.0492 ± 0.0079 nL/min, which represents over a three-fold greater secretion rate in comparison to unstimulated controls.

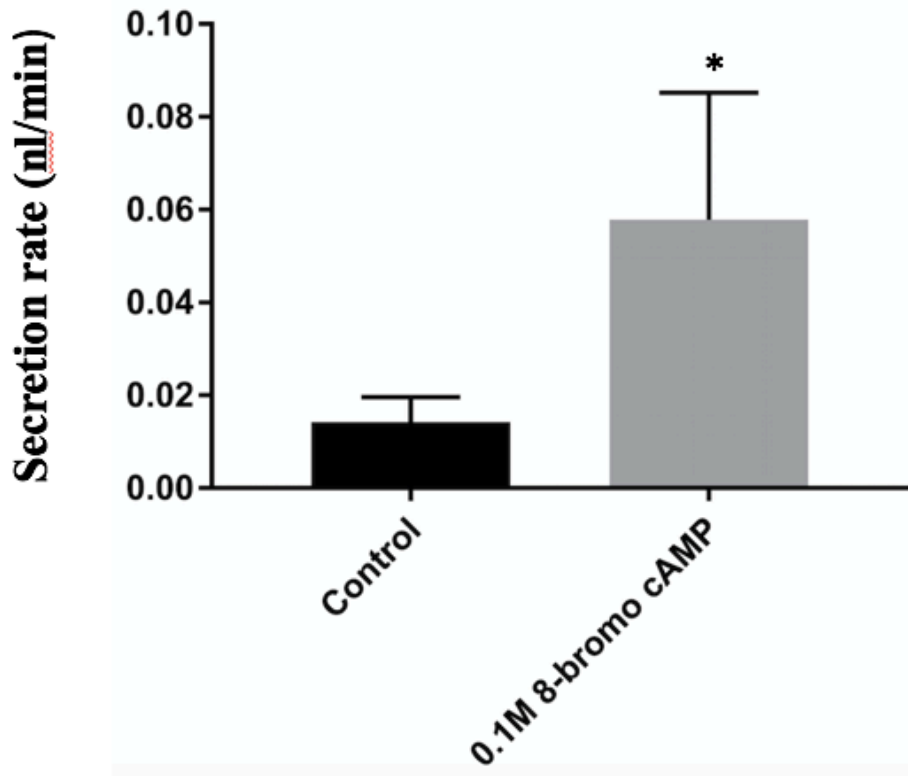


**Figure 15. Secretion rate of Malpighian tubules from *I. scapularis* in control saline for 30 minutes and in 0.1M 8-bromo cAMP for 30 minutes**. Control tubules shows an average secretion rate of 0.0142 ± 0.0054 nl/min whereas those treated with 0.1M 8-Bromo cAMP had an average secretion rate of 0.0579 ± 0.0273 nl/min. (n=20 per treatment). Standard error bars were calculated as standard error of the mean and were shown to be significant. Unpaired t-tests found differences between control and experimental treatments to be statistically significant ($p$<0.05).

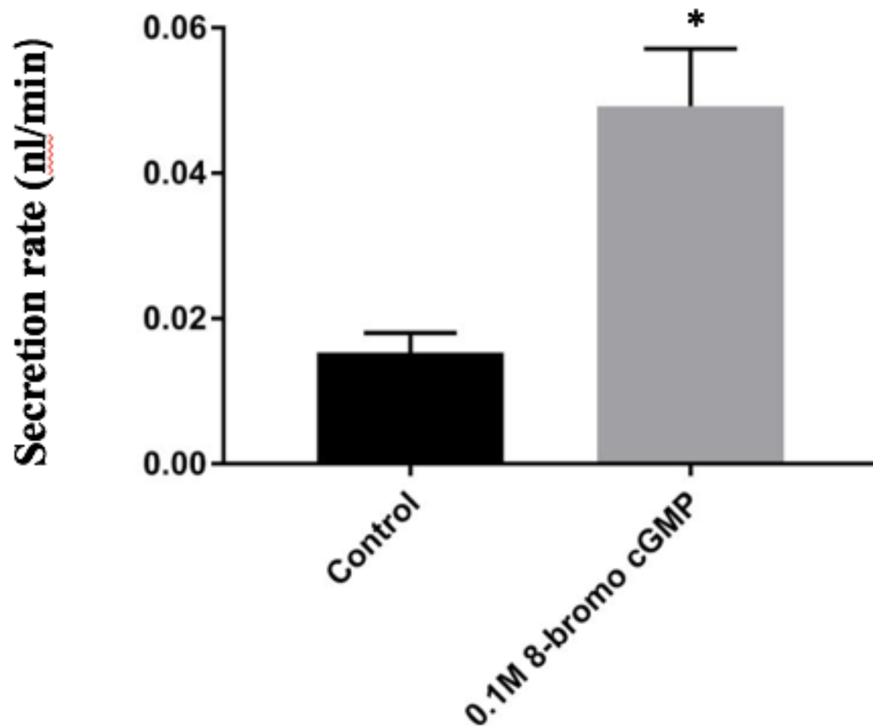


**Figure 16. Secretion rate of Malpighian tubules from *I. scapularis* in control saline for 30 minutes and in 0.1M 8-bromo cGMP for 30 minutes**. Control tubules shows an average secretion rate of 0.0153 ± 0.0027 nl/min whereas those treated with 0.1M 8-Bromo cGMP had an average secretion rate of 0.0492 ± 0.0079 nl/min. (n=16 per treatment). Standard error bars were calculated as standard error of the mean and were shown to be significant. Unpaired t-tests found differences between control and experimental treatments to be statistically significant ($p<0.05$).

NKA and VA Activity

NKA and VA activity were measured within gut regions in order to quantitatively determine the presence of these ATPases (Figure 17). Organs were isolated and pooled into tubes (n=5 samples), with 10 organ samples per tube. There were notable differences between NKA and VA activities (1μmol ADP $mg^{-1}$ protein $h^{-1}$) in males and females (Figure 17a). VA activity was higher in all organs in males in comparison to NKA however results were not significant ($p>0.05$). Of all three organs examined in males, the salivary glands showed the least NKA and VA activity, which is unusual considering their suggested dominant role in ion and osmoregulation. In females, VA and NKA activity was only found in the salivary glands, with higher VA activity in comparison to NKA (Figure 17b). All negative activity levels were zeroed for the activity assay. Student's paired t-test determined VA and NKA activity within the female salivary glands was significant different ($p<0.05$); however, ATPase activity in all other organs was inconclusive as no detectable activity levels were observed.

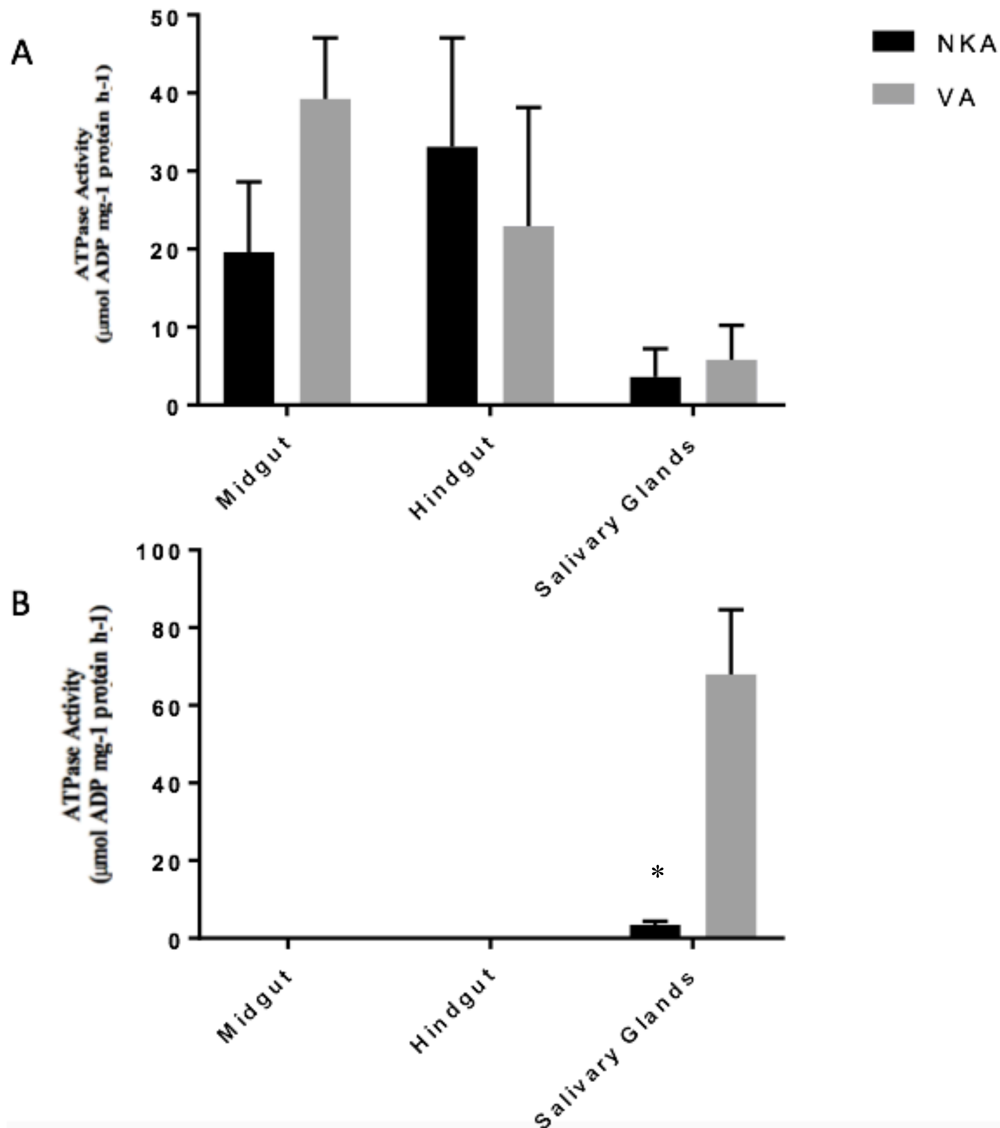


**Figure 17. $Na^+/K^+$ ATPase and V-type $H^+$ ATPase activity in *I. scapularis* midgut, salivary glands, and hindgut. (A)** Male NKA and VA activity in the midgut (MG), salivary gland (SG), and hindgut (HG). Data are expressed as mean ± SEM. (n=5, pool of 10). $p>0.05$. **(B)** Female NKA and VA activity in MG, SG, HG. Data expressed as mean ± SEM. (n=5, pool of 10). * denotes specific ATPase activity that was found to be significantly different ($p<0.05$).

## **Discussion**

Regulation of ions and fluids following blood meal engorgement and between bouts of blood feeding is critical to the survival of the black-legged tick, *Ixodes scapularis.* The salivary glands of *I. scapularis* have been implicated in the control of ion and osmoregulation during on-host feeding as the uptake of excess ions and water during blood meal engorgement is inevitable. However, the salivary glands are not the sole regulators working to regulate ion and fluid transport in this organism. Black-legged ticks are most commonly known as being the organism capable of transmitting *Borrellia burgdorferi* in North America, subsequently causing Lyme disease in humans after receiving a bite from an infected tick. Due to their critical role in pathogen transmission, research has been heavily focused on the salivary glands, specifically in the characterization of mechanisms involved in pathogen transmission. It is only recently that the characteristics of the salivary glands, which allow it to regulate ion and fluid levels during on-host feeding, have been looked at intently. Still, the role of other potential organs in ion and osmoregulation has been ignored and thus this investigation attempted to further characterize these other organs and determine their potential roles in ion and osmoregulation. The Malpighian tubules in other blood feeding arthropods, such as *A. aegypti,* are the major organs involved in alleviating the challenge of regulating osmotic and ionic concentrations in the haemolymph in relation to blood feeding. In this study, immmunohistochemical methods were utilized on the Malpighian tubules, midgut, hindgut, and salivary glands, to determine the distribution of two ionomotive pumps, the NKA and VA, which are known to facilitate ion and fluid transport across epithelia. Secretion assays were also used to determine the regulation of the Malpighian tubules, examining whether second messengers, such as cAMP or cGMP, can influence secretion rates. In order to quantify the functional contribution of NKA and VA in the above mentioned

organs, activity assays were completed. Ion and osmoregulation in *I. scapularis* are critical in maintaining homeostasis, and this study aimed to elucidate the potential role of the Malpighian tubules by examining their regulation, and to also further characterize the distribution of ionomotive pumps in the Malpighian tubules, midgut, hindgut, and salivary glands.

NKA and VA distribution in *I. scapularis* salivary glands

As the uptake of a blood meal progresses, salivary gland secretion rate increases allowing the tick to concentrate its blood meal and simultaneously return excess ions and water back to the host (Sauer 1984). In unstimulated salivary glands, NKA and VA are distributed in similar patterns between male and females *I. scapularis.* NKA immunoreactivity was present in all three types of acini in the salivary glands, but was differentially located. The type I acini presented the most abundant immunoreactivity of NKA, which is supported by recent work done by Kim et al. (2016) in which NKA was also found in type I acini towards the basolateral surface of individual cells. These findings are supported by the fact that type I acini are said to be involved in water vapor absorption via the secretion of a hygroscopic saliva during off-host periods (Kim et al. 2016). In type II acini, NKA immunoreactivity was observed in the abluminal epithelial cells, while in type III acini, it was found toward the periphery of each epithelial cell which is suggested to be the location where membrane folding forms a labyrinth (Kim et al. 2016). The type III acini are suggested to be the acini in which the most fluid and ions are excreted back to the host during blood meal engorgement, specifically f-cells which form an extensive basal labyrinth with the ablumenal interstitial cells during feeding (Sauer, Essenberg, and Bowman 2000). Co-localization of VA was found with NKA, specifically in the type III cells where, as previously described, ion and osmoregulation occur during on-host feeding. In the main ion and osmoregulatory tissues of *A. aegypti* (i.e. the Malpighian tubules),  VA drives ion transport

through the electromotive force of the pump by polarizing the apical membrane through $H^+$ transport from the cell into the tubule lumen, which allows for the transport of $K^+$ and $Na^+$ across the basolateral membrane (Wieczorek et al. 2000). Given that VA drives ion and osmoregulation in the Malpighian tubules in other blood feeding arthropods, it should be considered in potentially playing a secondary role to NKA in ion and osmoregulation in the type III acini during on-host feeding. However, given that VA has, until now, never been looked at in *I. scapularis*, further characterization is required in order to determine its specific role in ion and osmoregulation during both on host and off host periods.

Dopamine stimulation of the salivary glands caused differential distribution of NKA and VA between male and female *I. scapularis*. Previous work has shown that dopamine stimulates salivary gland secretion by acting on dopamine receptors D1 and InvD1L (Kim, Šimo, and Park 2014). In male salivary glands, the distribution of V-type $H^+$ ATPase increases after stimulation of dopamine. In female salivary glands, NKA distribution increases dramatically in the type III acini in comparison to unstimulated salivary glands. This supports previous studies in which it was determined that male salivary glands only secrete at $1/20^{th}$ of the rate of female salivary glands (Kaufman 1976). Females are larger than males and take up a larger blood meal, requiring that they regulate ion and fluid levels at a faster rate during on-host feeding. Previous studies suggested that NKA activation for electrochemical gradients may not be required for fluid transport in the type III acini cells, suggesting it may be involved in dopamine-mediated activation of aquaporins; however this idea is fairly novel and requires further investigation (Kim et al. 2016). Thus, VA immunoreactivity increasing due to dopamine stimulation could be due to differential roles in the acini type as male salivary glands are suggested to not play a significant role in ion and osmoregulation during on-host feeding (Sonenshine and Roe 2014). Unlike

females, males feed intermittently, attaching and detaching many times throughout the course of feeding. The salivary glands of males do not undergo the vast morphological changes that female salivary glands do. F-actin immunoreactivity was used as a marker for musculature and was found to be located in the alveolar duct of type II acini and in the adlumenal interstitial cell of the type III acini which borders the lumen. During blood meal engorgement, Type III acini are suggested to expand while fluid moves into the lumen and contract during secretion into the salivary ducts (Sonenshine and Roe, 2014).

NKA, VA f-actin distribution in *I. scapularis* midgut

NKA and VA were observed to be distributed throughout the midgut diverticula. NKA immunoreactivity is observed more intensely in the midgut diverticula in comparison to the main portion of the midgut (i.e. stomach) suggesting differential roles of NKA in regions of the midgut. VA immunoreactivity decreases toward the distal end of the midgut diverticula. Quantification of NKA and VA in the midgut was not significant. Activities of these enzymes were undetectable in females with our assay which could be suggestive of endogenous proteases reacting with inhibitors of NKA and VA, ouabain and bafilomycin. Unfortunately, no specific proteases or proteolytic enzymes which could be reacting with NKA and VA inhibitors have been characterized in the tick midgut to confirm this. Digestion of the blood meal occurs in the midgut epithelia along the diverticula, along with midgut pH balance. At the start of feeding, blood enters into the stomach (central portion) of the midgut, and then passes into the branched diverticula. Digestion occurs in the midgut epithelium in digestive cells (Sonenshine and Roe, 2014). Unfortunately, the physiological properties of the tick midgut are fairly uncharacterized and thus these findings are only suggestive of differential roles of the ionomotive pumps within different regions of the midgut. At the start of feeding, while the tick is attached, blood is passed

to the diverticula, in which excess ions and water then pass through the midgut epithelium into the haemolymph where they are removed primarily via the salivary glands (Sonenshine and Roe, 2014). VA localization differs along the anterior, mid anterior and posterior region of the *A. aegypti* midgut, driving luminal alkalization (Jagadeshwaran et al. 2010). In *A. aegypti*, the cells of the midgut in the adult female are found to express NKA basolaterally and VA apically, with the levels of expression of both being higher in the posterior midgut region (Patrick et al. 2006). This is supported by the notion that the posterior region of the midgut is suggested to have a function in ion and fluid transport, coinciding with the area in which blood meal waste accumulates before passage into the rectal sac via the intestine. F-actin immunoreactivity is presented in a circular pattern suggesting circular musculature allowing for segmentation of the midgut diverticula and thus the movement of food waste throughout the diverticula. The stomach has both circular and longitudinal musculature allowing for the mixing and digestion of food as well as propulsion of foodstuffs. Further insight into the role of the midgut in reabsorption of ions and fluid transport is required.

Localization of NKA, VA, and f-actin in the hindgut

Through immunohistochemical analyses, NKA was mainly observed in the intestine, while VA was observed in the rectal sac. Confocal images collected also showed guanine crystals in the intestine and rectal sac and thus NKA and VA distribution was not as visible. The majority of nitrogenous waste and $K^+$ is removed via the hindgut in ticks, while $Na^+$ is usually excreted back to the host during a blood meal by way of the salivary glands (Šimo and Park 2014). Guanine crystals (nitrogenous waste) are deposited from the Malpighian tubules into the rectal sac via the intestine for further concentration (Šimo and Park 2014). NKA activity quantified in the hindgut was higher in males in comparison to females, which could be

suggestive of the hindgut playing a more prominent role in ion and osmoregulation in males as opposed to the salivary glands, as males take up a smaller blood meal and subsequently, their salivary glands secrete less than females. In *A. aegypti*, reabsorption of ions and water in the primary urine occurs once it enters the hindgut (Coast, 2002). Thus, as *A. aegypti* are blood-feeding disease vectors like *I. scapularis*, the notion that reabsorption of ion and water occurs in the hindgut, driven by ionomotive pumps such as NKA and VA, should be considered. It was expected to find NKA and VA immunoreactivity throughout the hindgut. VA drives ion transport through ion specific channels while its electrochemical proton potential can allow for secondary active transport through cation or anion/$H^+$ exchangers/antiporters (Jonusaite, Kelly, and Donini 2011; Patrick et al. 2006). Previous studies on *Dermacentor andersoni* found that saliva excreted back to the host during blood meal engorgement contained more $Na^+$ than $K^+$, with excess ions being excreted via the anus and salivary glands. $K^+$ was found in excess in the rectal sac excretion compared to the excretion by the salivary glands (Kaufman and Phillips 1973). It is suggested that because $K^+$ is found heavily in the Malpighian tubule secretions of other insects, perhaps the Malpighian tubules of ticks are involved in secreting it, and thus supporting our findings of distribution of NKA and VA in the hindgut (Kaufman and Phillips 1973). Mechanisms of ion and osmoregulation within these organs have not been examined and thus further analysis is required before arriving at any concrete conclusions regarding ion and osmoregulation.

Malpighian tubules as potential tissues involved ion and osmoregulation

NKA and VA immunoreactivity was present throughout the Malpighian tubule length. VA immunoreactivity decreases towards the most distal end, suggesting differential roles of the ionomotive pump along the tubule length. Studies on *Rhodnius prolixus*, a blood feeding disease

vector, found that their Malpighian tubules excrete a hypo-osmotic fluid through which differential ion movement occurs in the distal and proximal Malpighian tubules (Maddrell and Phillips 1975). Due to findings in this study that demonstrated differential localization of NKA and VA along the tubule length, the idea that ion movement through the tubules will differ along the length is consistent with what is found in insects. The Malpighian tubules of *A. aegypti* are powered by VA which pumps protons into the tubule lumen, which creates an electrochemical gradient across the apical and basolateral membranes in principal cells. This in turn drives ion uptake from the haemolymph across the basolateral membrane through transporters and anion/cation antiporters (Piermarini, Esquivel, and Denton 2017). NKA in the basolateral membrane of the stellate cells is said to play a minimal role (Piermarini, Esquivel, and Denton 2017).

The membrane permeable analogs of two prospective second messengers, cAMP and cGMP, were observed to stimulate secretion by the Malpighian tubules in *I. scapularis.* Specifically, 8-bromo cAMP stimulated the tubules to secrete fluid at a rate over four-fold greater than the unstimulated control secretion rate. In insect Malpighian tubules, cAMP is a known second messenger of several peptides and hormones. In *A. aegypti,* mosquito natriuretic peptide (MNP) acts through the cAMP to stimulate secretion in the Malpighian tubules (Beyenbach 2003). Alone, cAMP duplicates effects of MNP, confirming its role as a second messenger (Beyenbach 2003). cAMP causes a depolarization of the basolateral membrane voltage simultaneously with a hyperpolarization of the transepithelial voltage. Along with this, the transepithelial resistance and the fractional resistance of the basolateral membrane decrease, with cAMP activating $Na^+$ levels at the basolateral surface (Beyenbach 2003). cAMP also activates $Na^+/K^+/2Cl^-$ cotransport which allows the entry of $Na^+$ into the cell, increasing $Na^+$ in

the cell and leading to its movement across the apical membrane facilitating transepithelial NaCl and water secretion. Additionally, dopamine stimulates salivary gland secretion in *I. scapularis* via dopamine receptors which activate cAMP pathways (Kim, Šimo, and Park 2014; Šimo et al. 2014). Thus, our findings that 8-bromo cAMP stimulate Malpighian tubule secretion are supported by similar studies done on the Malpighian tubules of *A. aegypti.* Stimulatory effects of 8-bromo cGMP were also observed, with the tubules increasing secretion rate by over three-fold. In *R. prolixus*, cGMP has been found to inhibit fluid secretion in the Malpighian tubules, and thus is considered to influence anti-diuresis (Quinlan, Tublitz, and O'Donnell 1997). In contrast, in *Drosophila melanogaster* Malpighian tubules, cGMP was found to have stimulatory effects (Davies *et al.* 1995). Thus, the results found in the current study on *I. scapularis* tubules differ from the effects of cGMP in *R. prolixus* tubules; however similar findings were found in *D. melanogaster*. cGMP may potentially affect the secretion rate at differing concentrations, and thus should be examined at low and high doses *in vitro* in order to characterize its potential inhibitory or stimulatory roles. In larval *A. aegypti*, cGMP is anti-diuretic at low concentrations (through the NOs/cGMP/PKG pathway), while is diuretic at high concentrations as it cross reacts with the cAMP pathway (Ionescu and Donini 2012). Unfortunately, the Malpighian tubules of *I. scapularis* are fairly uncharacterized and thus the current findings, although novel, have taken only initial steps to shed light on their regulation and function. Further studies and characterization of the tick Malpighian tubules are required to better understand their role in ion and osmoregulation as well as their hormonal regulation.

**Concluding remarks and future directions**

In summary, the distribution of NKA and VA varies across tick organs, most likely due to differential roles in ion and osmoregulation before, during and after blood meal engorgement.

The salivary glands are already recognized for NKA distribution within the different acini type; however, our findings suggest that the NKA distribution changes with dopamine stimulation, with differences occurring between males and females. Females excrete back excess ions and fluids during blood feeding at a higher rate than males, and thus comparatively, the salivary glands of males play a minimal role in ion and osmoregulation. This difference in sex affecting ion and osmoregulation should be further characterized as females thrive on blood meal engorgement, releasing nearly a thousand eggs after they blood feed as adults. VA is known to drive Malpighian tubule secretion in other blood feeding arthropods; however, its distribution and activity has not been previously described in *I. scapularis*. Our findings confirm that VA is distributed throughout the salivary glands and also undergoes changes in its distribution following dopamine stimulation of the salivary glands. Regarding other iono and osmoregulatory organs, such as the midgut, intestine, rectal sac, and Malpighian tubules, this study found that both NKA and VA are distributed among all organs examined. VA immmunoreactivity decreased towards the distal portion of the Malpighian tubules which could suggest that they share similar features to *R. prolixus* tubules in that segments of the tubule play different roles in ion regulation; however, further characterization is necessary. The Malpighian tubules were found to be sensitive to secretagogues, with stimulatory effects being observed by cyclic nucleotide membrane-permeable analogs, 8-bromo cAMP and 8-bromo cGMP. Due to the prevalence of *I. scapularis* as disease vectors, the majority of research has been focused on characterizing the physiological and biochemical mechanisms that allow for pathogen transmission. Given that, it is obvious that the iono and osmoregulatory processes in this organism are fairly understudied. The tick must be able to regulate excess ions and fluids that it takes up from its blood meal, and this process is crucial to its survival. It is suggested that further

studies be completed to determine the mechanisms underlying ion and osmoregulation within the salivary glands, rectal sac, and Malpighian tubules. Examining the effects of diuretic hormones on the tick Malpighian tubule can better help understand diuresis in this organism. Determining the exact mechanism of ion and fluid transport within these tissues is also beneficial. In better understanding the processes and organs responsible for ion and osmoregulation, there is potential for reducing pathogen transmission or directly limiting the distribution of *I. scapularis* populations.

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